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Functional Analysis of Abp1 in *Dictyostelium*

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Functional Analysis of Abp1 in *Dictyostelium*

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Dedication

To myself, my family and my friends

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Functional Analysis of Abp1 in *Dictyostelium*

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This work identified an ortholog of Abp1 (*a*ctin *b*inding *p*rotein 1) in *Dictyostelium* (Dabp1). In order to analyze the functions of Dabp1 in *Dictyostelium*, loss-of–function studies and gain-of-function studies were performed by generating cells that either deleted the Dabp1 gene from the genome or overexpressed the Dabp1 protein. In these mutants, most actin-based processes were intact. However, cell motility was altered during early development. During chemotactic streaming, more than 90% of wild type cells had a single leading pseudopodium and a single uropod, whereas more than 27% of Dabp1 null cells projected multiple pseudopodia. Similarly, ~ 90% of cells that overexpressed Dabp1 projected multiple pseudopodia during chemotactic streaming, and displayed reduced rates of cell movement. Expression of the SH3 domain of Dabp1 showed this domain to be an important determinant in regulating pseudopodium number. These results suggest that Abp1 controls pseudopodium number and motility in early stages of chemotactic aggregation in *Dictyostelium*.

This work also revealed an interplay between Dabp1 and MyoB, one of the Myosin I proteins, in controlling pseudopodia formation in *Dictyostelium*. These two proteins co-localize partially at the cortex in growing cells. The peripheral localization of MyoB was dependent on Dabp1. Depletion of both Dabp1 and MyoB caused defects in organization of the actin cytoskeleton and actin related activities such as formation of small F-actin filled spikes on the cell cortex of growing cells, a higher percentage of multinucleated cells, and an increased number of pseudopodia branching extensively. When MyoB was overexpressed in Dabp1 null mutants, cells had similar phenotypes as Dabp1/MyoB double null mutants, and displayed an increased number of pseudopodia with many branches. Overexpression of Dabp1 in MyoB null mutants rescued the defects in pseudopodia formation. The SH3 of Dabp1 was shown to be important for the rescue of defects caused by depletion of MyoB. Collectively, these data suggest that MyoB and Dabp1 work cooperatively to regulate the uniformity and integrity of the actin extensions during chemotaxis. MyoB requires Dabp1 to function in this process. Dabp1 may function as a scaffold to recruit MyoB to the proper localization.

These studies of Dabp1 in *Dictyostelium* raise broad question about functions of actin-associated proteins in pseudopodia formation and the importance of uniformity and integrity for actin structures in chemotaxis.

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Chapter 1 *Dictyostelium:* A Model System to Study the Actin Cytoskeleton during Chemotaxis

Actin cytoskeleton in *Dictyostelium*

The protein actin is highly conserved in all eukaryotic species and provides essential functions for eukaryotic cells. Generally, actin has two forms: monomeric actin (G-actin) and filamentous actin (F-actin). G-actin nucleates and polymerizes into F-actin to form many actin-based structures. These structures include the cell cortex, a thick layer of F-actin organized as a meshwork adjacent to the plasma membrane. The actin cortex functions to maintain the shape of the cells. By responding to different signals, cortical actin reorganizes and functions in different activities. For example, during macropinocytosis or phagocytosis, the cortical actin filaments quickly rearrange to extend cup-like structures that engulf liquid or particles. The captured cargo is then conveyed to the cell interior, forming membrane-bound early endosomes (Lamaze et al., 1997; Maniak, 2003). During chemotactic aggregation, the actin filaments organize to project a single dominant pseudopodium filled with a dense meshwork of F-actin at the leading edge (Affolter and Weijer, 2005). During cytokinesis, actin filaments and myosin motors organize a contractile ring that drives cleavage furrow formation (Burgess, 2005; Glotzer, 2005).

Due to frequent changes of cell shape, *Dictyostelium* can be used as an excellent model to study the rearrangements of the actin cytoskeleton and actin based activities such as phagocytosis, cell motility, and cytokinesis. Moreover, *Dictyostelium* cells have

two distinct life stages, a vegetative growing stage and a developing stage. Each stage is associated with different proteins that organize and regulate the actin cytoskeleton. Additionally, genetic manipulations such as knockout of target genes are relatively straightforward since *Dictyostelium* cells are haploid.

Because the actin cytoskeleton is involved in diverse cellular activities, eukaryotic cells must regulate the spatial and temporal polymerization, depolymerization and organization of actin filaments. Eukaryotic cells have developed several molecular mechanisms to regulate of the actin cytoskeleton.

First, the sequestration of G-actin provides a means to regulate actin. Inside the cell, the actin pool contains both G-actin and F-actin. When the concentration of G-actin is above the critical concentration, it will polymerize into F-actin. To control the formation of actin filaments, G-actin sequestering proteins bind to G-actin while capping proteins bind to the fast-growing end of F-actin to stop further elongation. In *Dictyostelium*, proteins such as thymosin β4 and profilin function as G-actin sequestering proteins while proteins such as CapZ and gelsolin function as capping proteins by binding the fast growing end of F-actin (Caldwell et al., 1989; Harris and Weeds, 1984; Kwiateck et al., 2000; Pantaloni and Carlier, 1993).

Secondly, cells regulate the selective depolymerization of F-actin. Depolymerization of F-actin is important in the rearrangements of actin. For example, the trailing tail of a moving cell, the uropod, must reorganize its actin filaments in order to retract (Iijima et al., 2002). Proteins such as destrin, actobindin and cofilin are most likely involved in

regulation of depolymerization of filaments (Bubb and Korn, 1995; Cvrckova et al., 2004; Nishida et al., 1985).

Third, cells regulate actin by nucleating new actin filaments. To polymerize G-actin into F-actin filaments, at least two mechanisms for nucleating actin have been proposed. One is that the Arp2/3 complex mediates actin nucleation while the other is that formin nucleates new actin filaments (Cvrckova et al., 2004; Evangelista et al., 2003; Machesky et al., 1994; Millard et al., 2004). The Arp2/3 complex was identified as a seven-protein complex localized to the leading edges of cells (Machesky et al., 1994; Machesky et al., 1997; Welch et al., 1997). The Arp2/3 complex caps the slow growing end of actin filaments and stabilizes the growing end (Zigmond, 1998). The Arp2/3 complex also binds to the sides of filaments and forms 70-degree branches (Welch et al., 1998). Formin proteins are a new family of proteins involved in actin nucleation (Pring et al., 2003; Zigmond, 2004). These proteins are highly conserved multi-domain proteins that play important roles in the formation of linear actin filaments, especially in formation of filopodia in *Dictyostelium* (Schirenbeck et al., 2005).

By itself, the Arp2/3 complex promotes the nucleation of F-actin only weakly (Mullins et al., 1998). Activator proteins such as WASP/SCAR can enhance the nucleation activities of the Arp2/3 complex (Ibarra et al., 2005). WASP has several protein interacting domains: a proline-rich domain that can interact with the actin binding protein profilin, Grb2 (Carlier et al., 2000; Chereau et al., 2005); a verprolin homology domain which can bind G-actin (David et al., 1998); a C-terminal acidic domain for the interaction with the Arp2/3 complex (Chereau et al., 2005; Kelly et al., 2006); a CRIB

(*c*dc42/*r*ac *i*nteractive *b*inding) domain for the interaction with small G proteins Rac and cdc42 (Rudolph et al., 1998). Therefore, WASP can link the Arp2/3 complex, Rac proteins and actin binding proteins such as profilin together for the polymerization of actin. SCAR is the homologue of WASP in *Dictyostelium* (Bear et al., 1998). This protein has similar functions as WASP in *Dictyostelium* cells (Ibarra et al., 2005).

Finally, cells regulate the organization of their actin cytoskeleton through signaling pathways. Among the binding partners of WASP/SCAR, Rac and cdc42 are small G-proteins involved in signaling pathways. This interaction between WASP/SCAR and small GTPases makes it possible to transmit outside signals to induce actin polymerization inside of the cell (Hall, 1998). To date approximately 15 Rac proteins have been identified in *Dictyostelium* cells (Bush et al., 1993; Rivero et al., 2001). Cellular activities that involve Rac GTPases include filopodia formation, cell motility during chemotaxis, macropinocytosis, and phagocytosis (Lee et al., 2003; Park et al., 2004; Rivero and Somesh, 2002). Additionally, they alternate between active form (GTP-bound) and inactive form (GDP-bound). Effectors such as GAP proteins or GEF proteins regulate the exchange of Rac proteins between active form and inactive form.

Collectively, many protein are involved in the regulation of actin polymerization. The actin cytoskeleton is maintained by the cooperation of all these proteins.

Chemotaxis in *Dictyostelium*

Chemotaxis is a process in which cells sense and respond to extracellular signals to adopt a polarized morphology and move in a chemical gradient. In *Dictyostelium*,

chemotaxis occurs early in development when individual ameobae respond to nutrient depletion by aggregating together and differentiating into a multicellular structure. During the process of chemotaxis, three major events are involved: sensing the extracellular signal, developing cell polarity and organizing the actin network for cell movement (Iijima et al., 2002).

During development, cells sense extracellular chemicals, and respond to these with intracellular signaling cascades (Parent and Devreotes, 1996). When nutrients are depleted, cells respond by secreting Cyclic AMP (cAMP), an extracellular chemoattractant. cAMP binds to the seven transmembrane cAMP receptor on the plasma membrane. Initially, these receptors are distributed randomly over the unpolarized cell surface (Ueda et al., 2001; Xiao et al., 1997). When cells experience a gradient of cAMP over the length of the cell body, a gradient of G protein activation is initiated by occupancy ratio of cAMP receptors (Janetopoulos et al., 2001). Downstream of the cAMP receptor, Gβ activates the small GTPase Ras and establishes a gradient of activated Ras proteins over the cell body (Kae et al., 2004). Subsequently, PI3 kinase, which has a Ras-binding domain and stays in cytosol in resting cells, is activated and recruited to the leading edge of the cell, where PI3 kinase converts PIP2 to PIP3 (Huang et al., 2003; Sasaki et al., 2004). At the same time, the phosphatase PTEN (phosphatase and tension homology) is prevented from binding to the leading edge but associates with the membrane at the rear of the cells in a Rho-dependent manner (Iijima and Devreotes, 2002). Possibly, Rho kinases activate PTEN at the rear by affecting its conformation or association with membrane. Since PTEN converts PIP3 to PIP2, prevention of PTEN

binding at the leading edge will decrease the conversion at this site. The cAMP-dependent actions, including the translocation of PI3 kinase to the leading edge, and the translocation of PTEN to the rear of the cell, result in an accumulation of PIP3 at the leading edge (Funamoto et al., 2002; Iijima et al., 2002). Since PIP3 can recruit a variety of PIP3 specific PH-containing proteins, a local concentration of activated kinases, such as Akt/Pkb, will accumulate at the leading edge (Chung et al., 2001; Dormann et al., 2004). When these kinases are activated, a spatial reorganization of the actin cytoskeleton can begin. In *Dictyostelium*, around 15 Rac proteins but no proteins with Rho homology have been identified (Rivero and Somesh, 2002). Accumulation of activated Rac proteins at the leading edge could locally increase actin polymerization (Chung et al., 2000). Thus the gradient of extracellular cAMP is essential for rearrangements of the actin cytoskeleton by affecting actin polymerization at the leading edge.

During chemotaxis, migrating cells maintain a relatively stable asymmetric shape with a distinct head and tail. A dynamic actin cytoskeleton contributes to the polarization of the cell (Gerisch et al., 1993a). Polarization is set up by the gradient of cAMP experienced by the chemotaxing cell. When the direction of the chemoattractant gradient changes, the polarization of the cell changes correspondingly to ensure that the leading region will always point to the high concentration of the gradient (Van Haastert and Devreotes, 2004). The old leading edge will disappear and a new leading edge will be generated in the new direction of the gradient. The advantage of polarization is to limit actin polymerization to the leading edge so that the cell moves persistently toward the aggregation center (Iijima et al., 2002). This is different from unpolarized cells, in which

the actin-filled pseudopodia extend randomly and, consequently, cell movement is slow and random.

Defects in cell motility can result in the prolonged aggregation of cells into mounds and the formation of smaller or abnormal fruiting bodies (Novak and Titus, 1997; Wang and O'Halloran T, 2006). Actin polymerizes at the leading edge to extend filopodia and lamilipodia, which generate major forces required for cell motility (Affolter and Weijer, 2005; Iijima et al., 2002). The retraction of the tail of aggregating cells is also very important for the motility. Actin depolymerization and the force generated by the contraction of myosin II filaments detach the tail region when cells move forward (Lauffenburger, 1996). Efficient motility is regulated by the coordinated rearrangements of the actin cytoskeleton at both the leading edge and the tail (Affolter and Weijer, 2005). One cellular structure that contributes to efficient motility is the pseudopodium. The number of pseudopodia, turnover of pseudopodia and a uniform shape for pseudopodia are important aspects for the regulation of pseudopodia. When cells polarize during chemotaxis, generally only a single dominant pseudopodium and one uropod are formed in each cell. An increase in the number of pseudopodia can impair *Dictyostelium* motility as cells aggregate during early development. For example, the *Dictyostelium* null mutants in MyoA and MyoB have increased lateral pseudopodia, and, as a consequence have reduced motility during aggregation (Wessels et al., 1991; Wessels et al., 1996). In addition to regulating the number of pseudopodia, cells also need to regulate the uniformity and integrity of pseudopodia. If the leading edge extension cannot stabilize into a pseudopodium, it will break into smaller actin structures. The resulting branches at

the leading edge can alter the region that senses the signal gradient leading to the loss of a dominant pseudopodium, and thus impairs cell motility. Studies in other species have shown that integrity of cellular extensions is critical to cell functions. In *Drosophila*, tricornered kinase and furry are important proteins to maintain the uniform shape of actin-filled projections of sensory neuron dendrites. Deletion of these proteins results in altered pattern of the dendritic fields (Cong et al., 2001; Emoto et al., 2004). However, to date it is not clear how *Dictyostelium* cells maintain the uniform shape of their actin-filled extensions such as pseudopodia.

Proteins involved in *Dictyostelium* chemotaxis

In order to successfully accomplish chemotaxis, cells must properly regulate three aspects of their behavior, including cell polarity, cell motility and signal sensing (Iijima et al., 2002). Particularly important aspect for cell motility is directional sensing because of its effect on actin polymerization and thus the rearrangement of the actin cytoskeleton. Cell polarity and cell motility are built on this fundamental process of directional sensing. To understand how cells regulate these three aspects, it is important to identity proteins that function in each process (**Table 1.1**).

Table 1.1 Proteins important for regulation of chemotaxis in *Dictyostelium*

Protein(s)	Localization and functions
Adenylyl cyclase (ACA)	Located at the tail region; production of cAMP; regulated by Ras and CRAC.
cAMP receptors(cAR1/cAR3)	Randomly distributed on cell membrane; G-protein coupled receptors containing seven transmembrane domains; cAR1 and cAR3 share redundant functions; cAMP binding to cAMP receptors results in persistent phosphorylation of the receptor and initiates signal cascade.
PI3 kinase/PTEN	PI3 kinase converts PIP2 to PIP3 while PTEN converts PIP3 to PIP2. PI3 kinase stays in cytosol in resting cells but translocated to the leading edge by binding to active Ras proteins. PTEN is depleted from the leading edge and accumulated at the tail region in a Rho dependent manner. The actions of the translocation and activation of PI3 kinase to the leading edge combines with the actions of the translocation and activation of PTEN to the tail in order to sharpen the localized activation of PIP3 at the leading edge. Both are important for the temporal and spatial regulation of PIP3.

G protein	(1) Gα: α subunit of G proteins; eight Gα have been isolated (Gα1-8). Gα2 coupled with cAR1 and cAR3. Mediate the cAMP dependent events in aggregating cells. (2) Gβ: β subunit; mediate all G protein dependent pathways, especially activation of Ras protein family.
PIP2/PIP3	Recruit a variety of PH-containing proteins to the leading edge; determine the position and formation of pseudopodia.
Proteins recruited by PIP2/PIP3	(1) Akt/Pkb: performs long distance regulation to PAKa. (2) CRAC (cytosolic regulator of adenylylcyclase): effecter of PIP3; regulates the activation and localization of ACA. (3) Rac: binds to SCAR and thus regulates the actin polymerization through the Arp2/3 complex; regulates the formation of pseudopodia.
Myosin	(1) Myosin II: localized in the back and sides of the migrating cells; stabilizes the polarity; generates forces for the traction of the tail; regulated by cGMP and the myosin heavy chain kinase, such as PAKa, in a PIP3 dependent manner. (2) Myosin I proteins: motor proteins of actin; generate contractile force to prevent the additional protrusions; regulate pseudopodium number.

PAKs (*p*21 *a*ctivated protein *k*inases)	(1) PAKa: regulates pseudopodia formation by affecting the phosphorylation of Myosin II at the tail region. (2) PAKc: cortical localization; regulates the phosphorylation of Myosin I proteins; regulates the formation of pseudopodia (3) MIHCK: regulates the phosphorylation of Myosin I proteins; regulates the formation of pseudopodia; works cooperatively with PAKc during chemotaxis.
Some major actin associated proteins	(1) SCAR/pir121: homologue of WASP; activator of Arp2/3 complex (2) Arp2/3 complex/Formins: nucleation of the actin polymerization

Myosin I family and Abp1 family

Myosin I proteins in *Dictyostelium*

Members of the myosin family have conserved motor domains that use the hydrolysis of ATP as an energy source to move along actin filaments. Amino acid homology searches have identified 12 myosin proteins classified into 6 groups: one conventional Myosin II (class II); seven myosin I proteins: MyoA-F and MyoK (class I); four other unconventional myosins: MyoH and Myosin J (class V or XI), MyoI (class XII) and MyoM (Soldati et al., 1999; Titus et al., 1994). These myosins are regulated by several different mechanisms: by calcium binding to the regulatory light chain; by

phosphorylation of the regulatory light chain; or by phosphorylation of a site in the motor domain of the heavy chain (Barylko et al., 2000; Brzeska and Korn, 1996; Gallagher et al., 1997; Szent-Gyorgyi et al., 1999). Phosphorylation of the heavy chains is particularly important for the activation of myosin I proteins (Barylko et al., 2000).

While Myosin II contains two heads with a motor domain, the seven myosin I proteins contain a single head adjacent to a variable tail. MyoA, MyoE and MyoF have short tails while MyoB, C and D have long tails. MyoK has no tail (de la Roche and Cote, 2001). The long tails of myosin B, C and D have three tail homology (TH) domains: a basic-rich TH1 domain, a glycine, proline and alanine-rich TH2 domain (also called GPA domain) and a Src homology 3 (SH3) domain (also called as TH3 domain). The short-tailed myosin Is have only a TH1 domain (de la Roche and Cote, 2001). MyoK is the smallest myosin. This protein has a very short neck region and a very short tail too, but the insert within the motor domain shares similarity to a TH2 domain (Schwarz et al., 2000; Yazu et al., 1999).

Along the tail region of Myosin I proteins, the basic-rich TH1 can bind membrane phospholipids while TH1 and TH2 can interact with actin filaments (Doberstein and Pollard, 1992; Jung and Hammer, 1994; Lee et al., 1999; Lynch et al., 1986; Rosenfeld and Rener, 1994; Schwarz et al., 2000). These domains suggest that the long-tailed myosin I proteins, such as MyoB, C and D, could be recruited to the membrane region by their TH1 domains, interact with actin filaments close to the membrane via their TH1 and TH2 domains, and pull actin filaments via their head motor domains. This is consistent with the immunofluorescence localization of long tailed myosin I proteins within sites of

dynamic actin, such as the leading edge, endocytic crowns and pseudopodia (Fukui et al., 1989; Jung et al., 1993; Jung et al., 1996; Morita et al., 1996).

The SH3 domain in the tail region is also important for MyoB, a long tailed myosin I. Deletion of MyoB or MyoA extends more lateral pseudopodia during chemotaxis (Titus et al., 1993; Wessels et al., 1991; Wessels et al., 1996); Deletion of both MyoA and MyoB or MyoB and MyoC exhibits decreased rates of pinocytosis and phagocytosis and has a lower cortical tension (Dai et al., 1999; Jung et al., 1996; Novak et al., 1995). However, overexpression of truncated MyoB with deletion of the SH3 domain exhibits a phenotype distinct from the overexpression of full-length wild type MyoB (Novak and Titus, 1997). The defects caused by deletion of MyoA and MyoB can be rescued by overexpression of wild type MyoB but not truncated mutant without the SH3 domain (Novak and Titus, 1998). These phenotypes suggest that the activity of Myosin I proteins may be linked to the actin based processes via their SH3 domains.

Since SH3 domains make highly specific interactions with PXXP motif, proteins containing proline-rich region are potential binding partners of long-tailed Myosin I proteins (Mayer and Gupta, 1998). In *Dictyostelium* cells, MyoB and MyoC use their SH3 domains to bind to the proline-rich region on scaffold protein p116/Acan 125, a protein that also binds capping protein (CP) and the Arp2/3 complex (Jung et al., 2001; Xu et al., 1997). Thus MyoB or MyoC can be coupled to the Arp2/3 complex through p116/Acan 125 and affects the actin assembly.

In order to perform their functions inside of the cells, Myosin I proteins must be activated by phosphorylating a single amino acid in the motor region of the heavy chain

(Brzeska et al., 1989; Lynch et al., 1989). This site is always a serine or threonine residue and is called a TEDS site (Bement and Mooseker, 1995). Myosin I heavy chain kinase (MIHCK), a member of p21-activated kinase family (PAK), functions to phosphorylate myosin I proteins in *Dictyostelium* cells (Brzeska et al., 1999; Lee and Cote, 1995; Lee et al., 1996). Besides MIHCK, myosin I proteins may also be phosphorylated by other PAKs or PAK-related kinases since the deletion of MIHCK doesn't impair the functions of myosin Is dramatically (de la Roche and Cote, 2001). Four members are now known in *Dictyostelium* PAK family: MIHCK, PAKa, PAKb and PAKc (Chung and Firtel, 1999; Chung et al., 2001; de la Roche and Cote, 2001; Lee et al., 2004). These proteins may share redundant functions.

In summary, myosin I proteins in *Dictyostelium* are thought to regulate the actin cytoskeleton and maintain a proper actin cortex by generating contractile force or by affecting the actin polymerization. In addition, myosin I proteins function in diverse actin-based processes such as pinocytosis, phagocytosis and pseudopodia formation. To function properly, Myosin I proteins need to be phosphorylated by PAK kinases to stay in an active form. Studies of Myosin Is, PAKs, and Myosin I-associated proteins are important in order to understand the functions of myosin I proteins.

Abp1 protein family

Abp1 is the first *a*ctin *b*inding *p*rotein identified in *Saccharomyces cerevisae* (Drubin et al., 1988). More recently, an ortholog of the yeast Abp1 was identified in mammalian cells, expanding the Abp1 protein family (Kessels et al., 2000). Members of the Abp1

family share an *a*ctin *d*epolymerizing *f*actor *h*omology domain (ADFH) at the NH2-terminus, a helical region in the middle, and a *S*rc *h*omology 3 domain (SH3) at the COOH terminus (Kessels et al., 2000; Lappalainen et al., 1998).

In yeast, Abp1 accumulates in the cortical actin patches (Drubin et al., 1988). These actin patches are enriched at the polarized cell surface during the formation of buds. When Abp1 is overexpressed, the actin cytoskeleton is altered and leads to an aberrant budding pattern and cortical actin assembly (Drubin et al., 1988). On the other hand, no apparent cytoskeletal defect is observed by deleting the *Abp1* gene (Lila and Drubin, 1997). However, deletion of Abp1 is synthetically lethal in combination with deletion of either Sac6p, Sla1p or Sla2p, which suggests that Abp1 shares redundant functions with these proteins (Cope et al., 1999; Drubin et al., 1990; Holtzman et al., 1993; Mayer and Eck, 1995). Moreover, this synthetic lethality cannot be rescued by Abp1 without its SH3 domain, indicating the importance of the SH3 domain in these protein interactions (Drubin et al., 1990). By using the SH3 domain to perform two-hybrid screens, six ligands have been identified for yeast Abp1. Two of these ligands, the Ser/Thr kinases, Prk1p and Ark1p, require Abp1 for their localization to actin cortical patches (Fazi et al., 2002). Prk1p is suggested to be a negative regulator of endocytosis, which links Abp1 to endocytosis machinery (Tang et al., 2000; Zeng and Cai, 1999; Zeng et al., 2001). Other ligands of Abp1 via its SH3 domain, such as Rvs167p/Rvs161p and synaptojanin/Inp52p, are also known to function in endocytosis (Lila and Drubin, 1997; Lombardi and Riezman, 2001; Ooms et al., 2000).

Abp1 contains two acidic DDW motifs, which have been shown to be important for the Arp2/3 binding and its function as a weak activator for the Arp2/3 complex during the nucleation of F-actin *in vitro*. The ADFH domain of Abp1 is essential for the binding of actin filament to Abp1 and is required for the Arp2/3 complex activation in vitro (Goode et al., 2001). Unlike WASP/SCAR, which activates actin nucleation by recruiting actin monomers to the Arp2/3 complex, Abp1 promotes filament nucleation by recruiting the Arp2/3 complex to the sides of actin filaments (Goode et al., 2001; Higgs and Pollard, 1999; Mullins, 2000). Taken together, these studies suggest that yeast Abp1 is not only important for the organization of the actin cytoskeleton, it also plays a role in endocytosis via the association mediated by its SH3 domain. It functions as a linker between protein complexes involved in actin polymerization and a number of proteins implicated during endocytosis (Olazabal and Machesky, 2001).

In mammalian cells, mAbp1 (the ortholog of yeast Abp1) localizes to the perinuclear region, but upon activation of the GTPase Rac, Abp1p translocates to the leading edge (Kessels et al., 2000). Although mAbp1 can accumulate at lamilipodia by responding to growth factors, it is not required for the formation of lamilipodia (Mise-Omata et al., 2003). mAbp1 contains two F-actin binding sites: an N-terminal ADFH domain and a central helical region. Both are important for F-actin binding to mAbp1 (Kessels et al., 2000). mAbp1 has been also implicated in endocytosis, because its SH3 domain associates with dynamin, a large GTPase essential for vesicle fission. Overexpression of the SH3 domain of mAbp1 has been shown to block receptor-mediated endocytosis (Kessels et al., 2001). Although the overexpression of the entire protein does not have

any effect on endocytosis, it inhibits protein trafficking at Golgi apparatus. Taken together, mAbp1 may also serve as a linker between receptor-mediated endocytosis and the actin cytoskeleton (Qualmann and Kessels, 2002).

Based on above studies, yeast Abp1 and mAbp1 share similar functions by acting as a linker between the actin cytoskeleton and one specific activity such as endocytosis. But each protein performs different mechanism to function in endocytosis or in regulation of the actin cytoskeleton. In yeast, Abp1 enhances actin polymerization by functioning as an activator of Arp2/3 complex. While mAbp1 binds F-actin, it is not clear how this protein affects the actin cytoskeleton. Mammalian Abp1 functions in endocytosis by binding to Dynamin, which generates the force to release vesicles from membrane. Knock down or overexpression of mAbp1 reduces endocytic activities. In yeast, Abp1 interacts with a different set of endocytic proteins, Rvs161p/Rvs167p, that also have diverse activities in events such as sporulation. Deletion of yeast Abp1 has no effect on endocytosis. The identification and study of Abp1 orthologs in *Dictyostelium* might help to reveal new contributions of Abp1 to the actin cytoskeleton and cellular activities. Moreover, studies of Abp1 proteins from different species will contribute to understand the evolution of one specific protein family.

Chapter 2 Abp1 Regulates Pseudopodium Number in Chemotaxing *Dictyostelium* Cells

Results described in this section were published in *Journal of Cell Science* (Wang Y. *et al.* 2006)

INTRODUCTION

During chemotaxis, cells polarize and coordinately move toward an extracellular signal. To effectively move, cells must detect shallow gradients of extracellular signal and translate this signal into changes in cell shape and cell adhesion (Parent, 2004). Particularly important for cell motility is the proper extension and retraction of actin-filled pseudopodia (Van Haastert and Devreotes, 2004; Williams and Harwood, 2003). Thus actin polymerization, depolymerization, and the branching and crosslinking of actin filaments must be under tight spatial and temporal control for effective cell movement (Iijima et al., 2002). To date, an increasing number of actin and actin-associated proteins have been identified that could regulate the organization of the actin cytoskeleton during directed chemotactic migration.

Dictyostelium discoideum is an excellent model to study chemotaxis and cell motility. When starved, *Dictyostelium* amoebae secrete pulses of extracellular cAMP and use this cue to stream together to form a multicellular aggregate. As they chemotax toward the cAMP signal, *Dictyostelium* amoebae rapidly extend and retract pseudopodia enriched in both actin and many actin-associated proteins. How these proteins coordinately regulate pseudopodia formation is not completely understood, but at least part of pseudopodium

behavior is accomplished by myosins. Two myosin I proteins, MyoA and MyoB, accumulate at the leading edge of streaming cells to regulate retraction of pseudopodia and to suppress non-productive pseudopodia formation (Novak et al., 1995; Wessels et al., 1996). The conventional myosin, Myosin II, concentrates at the trailing edge of chemotaxing cells, and functions to suppress lateral pseudopodia (Wessels et al., 1988). In addition to these myosins, actin-associated proteins also regulate actin dynamics within pseudopodium. The Arp2/3 complex, as well as its activators like the WASP/SCAR family of adaptor proteins, functions in actin network dynamics at the leading edge (Machesky and Insall, 1998; Mullins et al., 1998; Zalevsky et al., 2001). PIR21, a component of the SCAR complex, regulates actin polymerization and affects pseudopodia formation (Bear et al., 1998; Blagg et al., 2003). While some of the important regulators of pseudopodia formation are known, how their diverse activities are coordinated is not well understood.

One important regulator of the actin cytoskeleton in eukaryotic cells is the protein Abp1. Abp1 was one of the first actin binding proteins identified in the yeast *S. cerevisae* (Drubin et al., 1988). Overexpression of Abp1 in yeast alters actin organization and affects polarized cell growth (Drubin et al., 1990; Fazi et al., 2002). The mammalian Abp1 homologue functions in receptor-mediated endocytosis (Kessels et al., 2000; Kessels et al., 2001). Both yeast Abp1 and mammalian Abp1 contain an ADFH domain at their amino-termini that binds to actin. Both yeast and mammalian Abp1 also have an SH3 domain at their carboxyl-termini that binds ligands that function in endocytosis.

This domain structure supports a role for Abp1 as a functional link between the actin cytoskeleton and other proteins (Qualmann and Kessels, 2002).

While Abp1 is found in a diverse array of organisms, the contribution of Abp1 activity to cellular functions remains an open question. Here we report on the contribution of Abp1 to cellular function in *Dictyostelium*. We find that Dabp1 (*Dictyostelium a*ctin *b*inding *p*rotein 1) is concentrated in dynamic regions of the cell cortex rich in F-actin. Moreover, *Dictyostelium* cells engineered to either delete or to increase expression of the Dabp1 protein displayed a distinct phenotype. Altering levels of Dabp1 in cells profoundly impaired the ability of cells to limit pseudopodium number in early development, and limited rates of directed cell movement. Consequently, cells overexpressing Dabp1 formed smaller aggregation centers and small fruiting bodies during development. These results reveal a specific and critical role for Dabp1 in regulation of pseudopodium number during directed cell migration.

RESULTS

Dabp1 is a member of the Abp1 family

Searching the *Dictyostelium* Gene Database (www.dictybase.org) revealed a single gene for Abp1, (accession number: AY437927, *Dictyostelium abpE*) housed on chromosome 2. The predicted reading frame for the Dabp1 gene, *abpE*, encoded a protein of 481 amino acids with a molecular mass of 59 kilodaltons. Similar to Abp1 proteins from other species, the amino acid sequence of the Dabp1 protein included an ADFH (*a*ctin *d*epolymerizing *f*actor *h*omology) domain at the amino-terminus (amino

acid 1 to 130), and an SH3 (*S*rc *h*omology 3) domain at the carboxyl-terminus (amino acid 422 to 481). Relative to the rest of the protein, these domains shared a high degree of amino acid identity with other abp1 proteins. Across its entire length, Dabp1 shared 21% identity with mouse Abp1 and 18% identity with Abp1 in *S. cerevisae*. The ADFH domain in Dabp1 shared 28.5% identity with mouse Abp1 and 19.2% identity with the *S. cerevisae* Abp1. The SH3 domain was more conserved among Abp1 members. The SH3 domain in Dabp1 shared 37% identity with mouse Abp1 and 34% identity with Abp1 in *S. cerevisae* (**Fig. 2.1**). Using a PCR-based approach, we cloned a cDNA for the gene and generated an antibody against the gene product. Western blots probed with affinity-purified antibodies raised against the *E.coli*-expressed fusion protein revealed that the Dabp1 protein migrated anomalously on SDS-gels with a molecular mass of 70 ± 3 kilodaltons (**Fig. 2.2**). While cells overexpressing Dabp1 tagged with GFP showed multiple bands in western blots, cells that overexpressed Dabp1 without the GFP tag did not display multiple bands (data not shown).

Dabp1 is enriched in the cell cortex and the leading edge

To determine the intracellular location of Dabp1, we stained *Dictyostelium* cells with affinity-purified anti-Dabp1 antibodies. Inspection of growing cells by fluorescence microscopy revealed an extensive association of Dabp1 with the cell cortex (**Fig. 2.3, top panels**). In polarized cells undergoing directed migration during early development, Dabp1 was especially concentrated at the leading edge (**Fig. 2.3, bottom panels**). To investigate a possible association of Dabp1 with the actin cytoskeleton, we stained cells simultaneously with phalloidin to label F-actin and with antibodies against Dabp1. In

growing cells, Dabp1 co-localized with F-actin at some, but not all, portions of the cortex (**Fig. 2.3, top panel**). In streaming cells, Dabp1 co-localized with F-actin to a much higher extent and completely overlapped with actin at the leading edge (**Fig. 2.3, bottom panel**). We observed that the amount of Dabp1 varied in growing cells. One possible reason is that Dabp1 associates only with portions of actin cytoskeleton that are particularly dynamic, a state that might differ between cells. In contrast Dabp1 consistently associated with the leading edge of migrating cells, a region of active actin remodeling, suggesting that Dabp1 could associate preferentially with areas of dynamic F-actin in *Dictyostelium*.

An intact actin cytoskeleton is required for the cortical residence of Dabp1

The localization of Dabp1 at the cell cortex suggested that the actin cytoskeleton could be required for the cortical localization of Dabp1. To test this idea, we disrupted the filament-rich actin cortex in wildtype cells with cytochalasin A, a drug that depolymerizes F-actin. Subsequently staining F-actin cells with TXRED-labeled phalloidin confirmed that cytochalasin dramatically altered cortical actin. Labeling the cytochalasin-treated cells with an antibody against Dabp1 showed that the association of Dabp1 with the cell cortex was abolished concurrent with the loss of cortical actin (**Fig. 2.4A**). These results indicated that cortical actin was required for the cortical localization of Dabp1.

Deletion or overexpression of Dabp1 has no effect on the cortical localization of actin

To examine a possible requirement of Dabp1 for the cortical localization of actin, we generated cell lines with altered expression of the Dabp1 protein. Using homologous recombination, we deleted the *abpE* gene to generate Dabp1 null mutants (Dabp1$^-$ cells). Cell lines with increased levels of Dabp1 (Dabp1$^+$ cells) were also made by transforming both a wildtype strain and the Dabp1 null mutants with an extrachromosomal plasmid that overexpressed GFP-Dabp1 fusion protein. Western blots probed with an anti-Dabp1 serum confirmed the absence of the Dabp1 protein in Dabp1$^-$ cells, and increased levels of Dabp1 protein in Dabp1$^+$ cells (**Fig. 2.2**). Initial phenotypic analysis of these strains showed that the absence of Dabp1 did not influence cell growth, while the overexpression of Dabp1 retarded cell growth slightly.

To examine the influence of Dabp1 on cortical actin, we stained Dabp1$^-$ cells and Dabp1$^+$ cells with TXRED-labeled phalloidin. Inspection of growing cells by fluorescence microscopy revealed that neither the absence nor increased levels of Dabp1 protein altered the cortical organization of the actin cytoskeleton (**Fig. 2.4B**). Moreover, after treatment with cytochalasin and subsequent washout, Dabp1$^-$ and Dabp1$^+$ cells reestablished localization of actin to the cortex with kinetics similar to wildtype cells (data not shown).

Overexpression of Dabp1 impedes aggregation of streaming cells

The association of Dabp1 with the leading edge of developing cells suggested that Dabp1 could function in areas containing dynamic actin. Active remodeling of the actin

cytoskeleton is particularly important when *Dictyostelium* amoebae develop into multicellular fruiting bodies in response to starvation. During early development, *Dictyostelium* cells respond to extracellular cAMP signals and coordinately stream into aggregation centers (Gerisch, 1987). Actin and actin-associated proteins form a dynamic gel organized into a dominant pseudopod at the leading edge of polarized cells (Gerisch et al., 1993b). We therefore tested a possible contribution of Dabp1 to the ability of cells to organize their actin into pseudopodia, adopt a polarized morphology, and move efficiently during development. Wildtype cells, Dabp1$^-$ cells, and Dabp1$^+$ cells were placed under starvation buffer to induce chemotaxis into aggregation centers.

Under these conditions, wildtype cells readily adopted a polarized shape (**Fig. 2.5, left panels**). By 12-13 hrs, streams of wildtype cells moving into an aggregation center were readily apparent. By 18 hrs, most wildtype cells were integrated into either streams or large aggregation centers. Dabp1$^-$ mutants followed a similar developmental pattern: by 8 hrs, cells were elongated, and by 13 -18 hrs Dabp1$^-$ cells were incorporated into streams centered around an aggregation center (**Fig. 2.5, middle panels**). In contrast, Dabp1$^+$ cells were dramatically delayed in early development. Dabp1$^+$ cells were unable to adopt a polarized morphology efficiently; elongated cells were not seen until 13 hrs under starvation buffer (**Fig. 2.5, right panels**). Small aggregation centers surrounded by broken streams of cells were only apparent after 18-19 hrs.

To examine later developmental stages, we placed equal numbers of wildtype cells, Dabp1$^-$ cells, and Dabp1$^+$ cells on nonnutrient agar plates, a surface on which *Dictyostelium* cells complete development to form multicellular fruiting bodies consisting

of a spore-filled sorus on top of an elongated stalk. After 14 hours on nonnutrient plates, wildtype cells and Dabp1$^-$ cells had formed multicellular migrating slugs. At 14 hours, most Dabp1$^+$ cells had only aggregated into mounds, an earlier stage of development (**Fig. 2.6**). By 28 hours, both wildtype cells and Dabp1$^-$ cells were fully developed into robust fruiting bodies consisting of a stalk topped with a sorus full of spores. In contrast, Dabp1$^+$ cells took about 12 hours longer to develop and formed much smaller fruiting bodies (**Fig. 2.6**). Thus overexpression of Dabp1 resulted in delayed aggregation of streaming cells, formation of smaller aggregation centers and smaller fruiting bodies.

Dabp1 influences polarity and pseudopodium number in streaming cells during chemotaxis

Light microscopy was used to monitor wildtype cells, Dabp1$^-$ cells and Dabp1$^+$ cells during chemotaxis (**Fig. 2.7**). As they streamed together, wildtype cells adopted a highly elongated and polarized morphology. The average length of polarized wildtype cells was 28.1 ± 3.1 μm (n=30). Dabp1$^-$ cells were also elongated in early development, and similar in average length to wildtype cells (26.2 ± 3.3 μm; n=12). However Dabp1$^+$ cells failed to adopt an elongated shape during early development. The average length of the streaming Dabp1$^+$ cells was 18.9 ± 2.0 μm (n=37), only 67% of the length of streaming wildtype cells.

In addition to decreased cell length, cells that overexpressed Dabp1 also displayed an increase in pseudopodium number. As they chemotaxed, wildtype cells generally formed two protrusions from the cell body, a dominant pseudopodium at the leading edge and a trailing uropod at the rear. Approximately 92% wildtype cells exhibited this morphology

(**Table 2.1**). Of the remaining 7.5% of wildtype cells, none had more than 5 cellular protrusions. The number of pseudopodia increased somewhat in three independent Dabp1$^-$ cell lines: 26.5% of the null mutants projected multiple pseudopodia; none had more than 5 pseudopodia. By contrast, Dabp1$^+$ cells displayed a dramatic increase in pseudopodium number. The majority of cells (~90%) overexpressing Dabp1 had multiple pseudopodia (**Table 2.1**), with some cells exhibiting as many as 10 pseudopodia. The stellate shape of Dabp1$^+$ cells made it difficult to discern a distinct leading pseudopodium and or a trailing uropod. Imaging chemotaxing cells showed that wildtype cells generally extended a single pseudopodium persistently in the direction of movement. Rather than projecting a dominant pseudopodium, most Dabp1$^+$ cells extended pseudopodia in multiple directions. Moreover, we also noticed that the extension and retraction of pseudopodia was slower in the Dabp1$^+$ cells, resulting in slower turnover of pseudopodia relative to wildtype cells (**supplementary material Movie 1, http://jcs.biologists.org/cgi/content/full/119/4/702/DC1)**. An increased number of pseudopodia extended in multiple directions were also observed in cells overexpressing Dabp1 without the GFP tag, indicating that the phenotype was not due to the addition of the GFP tag to Abp1 (**supplementary material Movie 2, http://jcs.biologists.org/cgi/content/full/119/4/702/DC1)**. The impaired ability to extend a dominant pseudopodium in a single direction may explain why Dabp1$^+$ cells take longer to stream into aggregation centers.

The pseudopodia formed by wildtype cells are enriched in filamentous actin. The deletion of Abp1 had a small effect on pseudopodium number while overexpression of

Abp1 resulted in a significantly increased number of pseudopodia in chemotaxing cells. To determine whether these pseudopodia were also enriched in F-actin, we stained cells with TXRED-labeled phalloidin **(Fig. 2.8**). Chemotaxing wildtype cell formed a single pseudopodium at its leading edge enriched in F-actin. While Dabp1$^-$ and Dabp1$^+$ cell formed multiple pseudopodia, each pseudopodium was enriched normally in F-actin.

Dabp1 impedes cell motility

The delayed aggregation and altered morphology exhibited by Dabp1$^+$ prompted us to examine whether these cells also displayed defects in motility. To initiate chemotaxis, we submerged wildtype cells, Dabp1$^-$ cells and Dabp1$^+$ cells under starvation buffer. When the cells were actively streaming, we imaged independent cells moving toward aggregation centers. Wildtype cells moved persistently in one direction (**Fig. 2.9A, supplementary material Movie 3, http://jcs.biologists.org/cgi/content/full/119/4/702/DC1**). Approximately 90% of movements were greater than 8 μm/min, with an average velocity of 12.5 ± 6 μm/min (n=159, 15 independent cells) (**Fig. 2.9B**). Dabp1$^-$ cells also moved persistently in one direction (**Fig. 2.9A**, **supplementary material Movie 4, http://jcs.biologists.org/cgi/content/full/119/4/702/DC1**), with around 80% of the movements were greater than 8 μm/min, and an average velocity of 11.1 ± 6 μm/min (n=129, 11 independent cells) (**Fig. 2.9B**). In comparison, Dabp1$^+$ cells frequently stalled in one location without productive translocation (**Fig. 2.9A, supplementary material Movie 1, http://jcs.biologists.org/cgi/content/full/119/4/702/DC1**). When Dabp1$^+$ cells translocated, only 34% of their movements were greater than 8 μm/min, and their average

velocity was only half that of wildtype cells (6.8 ± 4 μm/min; n=166, 12 independent cells) (**Fig. 2.9B**).

The SH3 domain is important for the morphology of streaming cells during aggregation

The Dabp1 protein has an ADFH domain at its amino-terminal domain and an SH3 domain at its carboxyl-terminus. To test the functional contribution of these domains to Dabp1, we designed two plasmids to express either the ADFH domain or the SH3 domain (**Fig. 2.10A**). Each domain was expressed as a fusion protein with GFP at the amino-terminus. The expression plasmids were introduced into wildtype cells. Western blots probed with anti-GFP antibodies showed high expression levels for the two GFP-tagged proteins (data not shown).

To examine the influence of these domains on cell phenotype, cells expressing the ADFH domain ($ADFH^+$ cells) and cells expressing the SH3 domain ($SH3^+$ cells) were induced to begin chemotaxis by starvation. $ADFH^+$ and $SH3^+$ cells formed normal fruiting bodies when plated on non-nutrient agar. Nonetheless, overexpression of the SH3 domain dramatically altered the morphology of cells during streaming in early development (**Fig. 2.10B**). In contrast with $ADFH^+$ cells, which appeared similar in morphology to wildtype cells, most streaming $SH3^+$ cells exhibited multiple pseudopodia (**Table 2.1**). Cells overexpressing SH3 domain exhibited up to 7 pseudopodia during chemotaxis. Thus the SH3 domain appeared to be a key functional determinant in controlling pseudopodium number during the aggregation phase of early development.

DISCUSSION

In early development, chemotaxing cells rapidly rearrange their actin cytoskeleton in order to adopt an effective shape for cell movement (Pollard and Borisy, 2003; Williams and Harwood, 2003). Remodeling the actin cytoskeleton results in extension of a dominant pseudopodium at the leading edge and retraction of the uropod at the trailing edge, coordinated shape changes that allow cells to move in response to a extracellular chemical gradient (Varnum-Finney et al., 1987). Key to productive motility is the tight control of pseudopodium number for cells moving toward an aggregation center (Chung and Firtel, 2002). Here we have shown that overexpression of Dabp1 resulted in formation of multiple pseudopodia and impaired cell motility during early development. These defects likely account for the delay in forming aggregation centers by cells overexpressing Dabp1. Because of their inability to form a single dominant pseudopodium, Dabp1$^+$ cells frequently stalled while extending pseudopodia in multiple directions, displayed reduced cell velocities, and failed to move persistently to an aggregation center. As a consequence of impaired motility, cells overexpressing Dabp1 formed smaller aggregation centers on starvation plates and made small fruiting bodies.

A concern with interpreting overexpression experiments is that phenotypes resulting from overexpression of a protein may not reflect the normal function of the protein. Nonetheless, the similarity in phenotypes exhibited by Dabp1$^+$ cells and Dabp1$^-$ cells supports the interpretation that the phenotypes associated with overexpression of Dabp1 reveal its physiological role. Both Dabp1$^+$ cells and three independent Dabp1$^-$ cell lines projected excess pseudopodia during chemotaxis. Overexpression of Dabp1 yielded

stronger phenotypes: relative to Dabp1$^-$ cells, Dabp1$^+$ cells exhibited an increased number of pseudopodia, and also displayed a dramatic reduction in cell motility. The milder phenotype associated with Dabp1$^-$ cells could reflect redundancy in proteins that regulate the actin cytoskeleton: when Dabp1 is depleted, other proteins could substitute partially for its function. Functional redundancy during development has been appreciated previously for other proteins associated with the actin cytoskeleton (Witke *et al.*, 1992).

Formation of extra pseudopodia was limited to developing Dabp1$^+$ cells; growing cells that overexpressed Dabp1 were normal in appearance. This intracellular role for Dabp1 was also specific for pseudopodia formation and not general for other actin-based processes. Most other actin-based processes were intact in the Dabp1 null mutants, including cytokinesis, pinocytosis and phagocytosis (data not shown). Pseudopodia in streaming cells contain abundant amounts of F-actin and the pseudopodia in Dabp1$^+$ cells were similarly rich in F-actin. Thus Dabp1$^+$ cells were able to construct pseudopodia normal in appearance, but were defective in limiting the number of pseudopodia during development.

A repertoire of proteins important for regulation of pseudopodium number is emerging. One class of proteins known to regulate pseudopodium number in *Dictyostelium* is unconventional myosins. MyoA and MyoB localize to the leading edge of streaming cells and play critical roles in regulating where and when a cell forms pseudopodia (Morita et al., 1996; Titus et al., 1993; Wessels et al., 1996). These two myosin Is play independent roles in suppressing lateral pseudopodia formation during chemotaxis (Falk et al., 2003). Null mutants for either MyoA or MyoB extend a greater

number of pseudopodia. Overexpression of MyoB inhibits pseudopodia formation and impairs cell motility (Novak and Titus, 1997). Like myosin I proteins, Dabp1 was enriched in pseudopodia, and conceivably could influence pseudopodium number by participating in a regulatory pathway involving MyoA or MyoB. Dabp1 could also contribute to other pathways that regulate polymerization of actin in pseudopodium. For example, Dabp1 could influence the activity of a protein that promotes actin polymerization, like Arp2/3, also localized in pseudopodium.

How might Dabp1 influence the activity of another regulatory protein? The phenotype resulting from overexpression of domains of Dabp1 favors the idea that Dabp1 binds a regulatory protein via the carboxyl-terminal SH3 domain. Overexpressing the ADFH domain of Dabp1 had little effect on the morphology of chemotaxing cells. In contrast, overexpression of the SH3 domain resulted in an increased number of pseudopodia in chemotaxing cells, similar to the defect seen in cells overexpressing full–length Dabp1. This dominant negative phenotype suggests that the Dabp1 protein influenced pseudopodium number through binding partners for the SH3 domain. Binding partners for the SH3 domain of Abp1 homologues in other species have also been found to be important for its function. The SH3 domain of mammalian Abp1 binds to the GTPase dynamin, and overexpression of this SH3 domain in cultured cells causes defects in receptor-mediated endocytosis (Kessels et al., 2001). The SH3 of yeast Abp1 binds to six ligands, all proteins involved in endocytosis (Fazi et al., 2002). Conceivably, overexpression of the SH3 domain of Abp1 could sequester partners for all of the SH3 domains involved in multiple pathways. However, overexpression of the SH3 domain of

Abp1 probably sequesters a limited set of discrete ligands. Different SH3 domains are sufficiently specific to distinguish subtle differences in the primary structure of potential ligands (Rickles et al., 1995; Sparks et al., 1996). Thus, it seems more likely that the dominant negative effect of overexpression of the SH3 domain is due to binding a proline-rich ligand important for limiting pseudopodium number in chemotaxing cells.

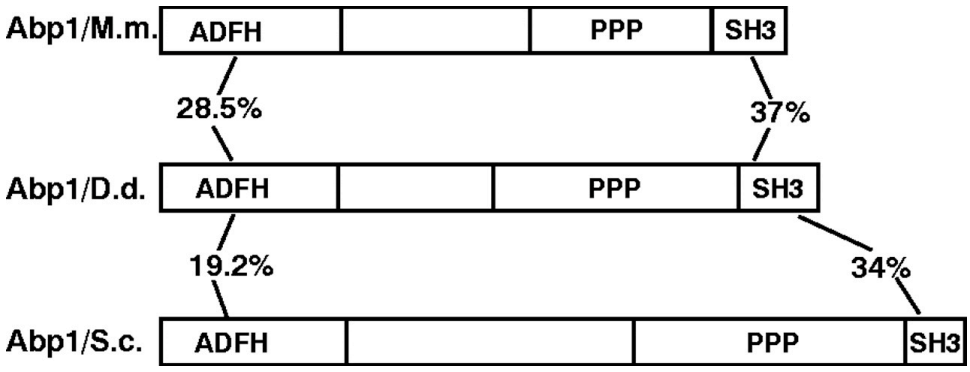

Figure 2.1 Domain structures of Abp1 orthologs in different species. M.m., *Mus musculus;* S.c., *Saccharomyces cerevisiae*; D.d., *Dictyostelium discoideum*; ADFH, actin depolymerizing factor homology domain; PPP, proline-rich region; SH3, Src homology 3 domain. Numbers indicate amino acid identity shared between domains of abp1 orthologs.

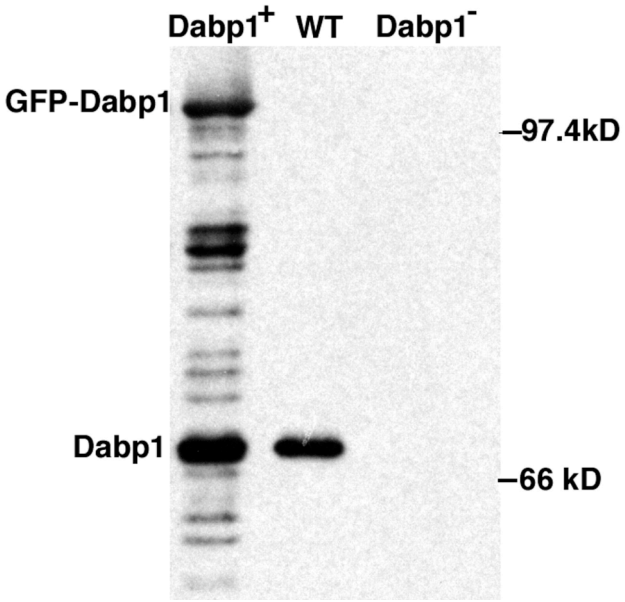

Figure 2.2 Knockout and overexpression of Dabp1. A western blot of whole cell lysates from $2.5x10^5$ wildtype cells (WT), Dabp1 null cells ($Dabp1^-$) and cells overexpressing Dabp1 ($Dabp1^+$) was stained with affinity-purified anti-Dabp1 antibodies.

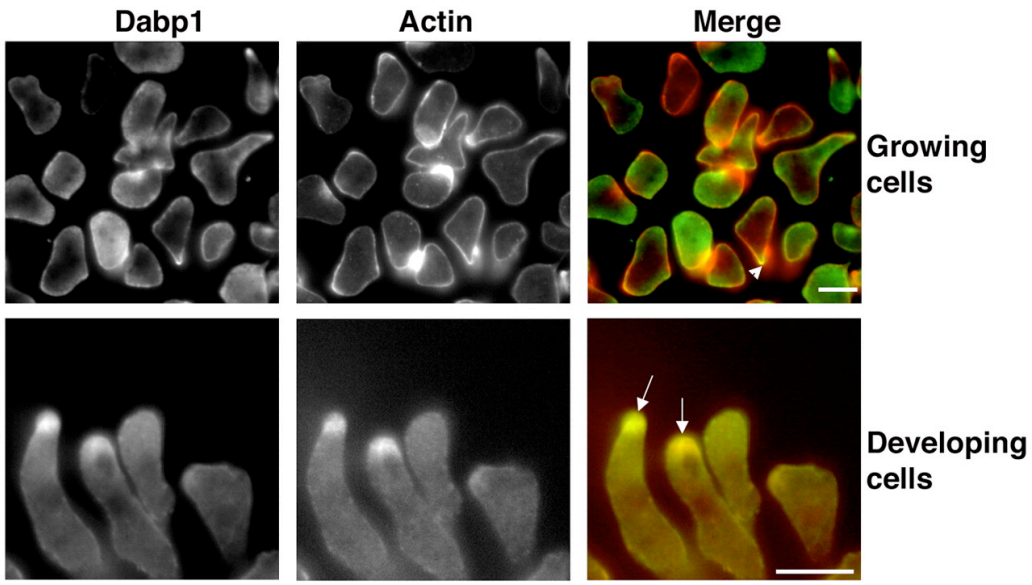

Figure 2.3 Dabp1 is enriched in the cortex and the leading edge of chemotaxing cells. Growing cells (top panels) and developing cells (bottom panels) were fixed and stained with affinity-purified anti-Dabp1 antibodies followed by BODIPY-FL-labeled secondary antibodies (to detect Dabp1) and TXRED-labeled phalloidin (to detect actin). Arrows and arrowhead show areas where Dabp1 and actin co-localize (in the merged images). Bar, 10 μm.

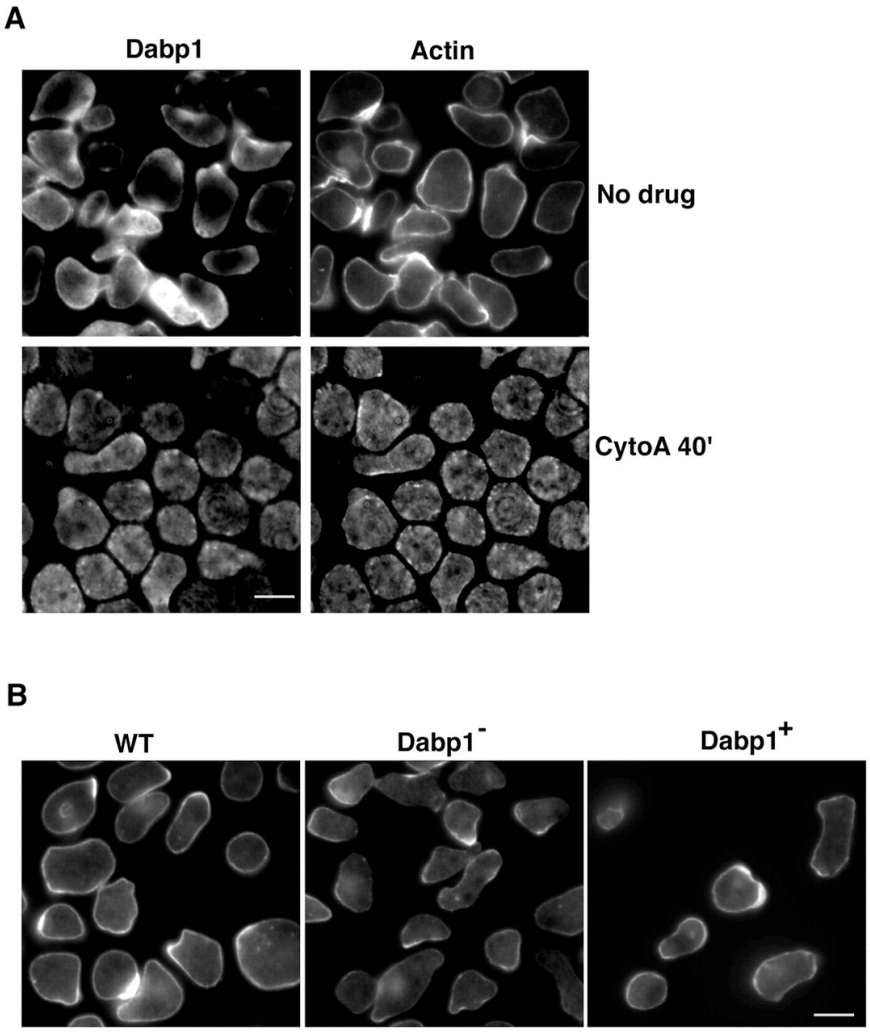

Figure 2.4 Dabp1 is not required for proper organization of the actin cytoskeleton. (A) The cortical localization of Dabp1 requires an organized actin cytoskeleton. Wildtype cells were treated with 10 μM cytochalasin A for 40 minutes and then stained with affinity-purified anti-Dabp1 antibodies (Dabp1) and TXRED-labeled phalloidin (actin). (B) Neither the absence of, nor an increase in, Dabp1 protein influences the actin cortex. Wildtype cells (WT), Dabp1 null cells ($Dabp1^-$) and cells overexpressing Dabp1 ($Dabp1^+$) were fixed and stained with TXRED-labeled phalloidin. Bar, 10 μm.

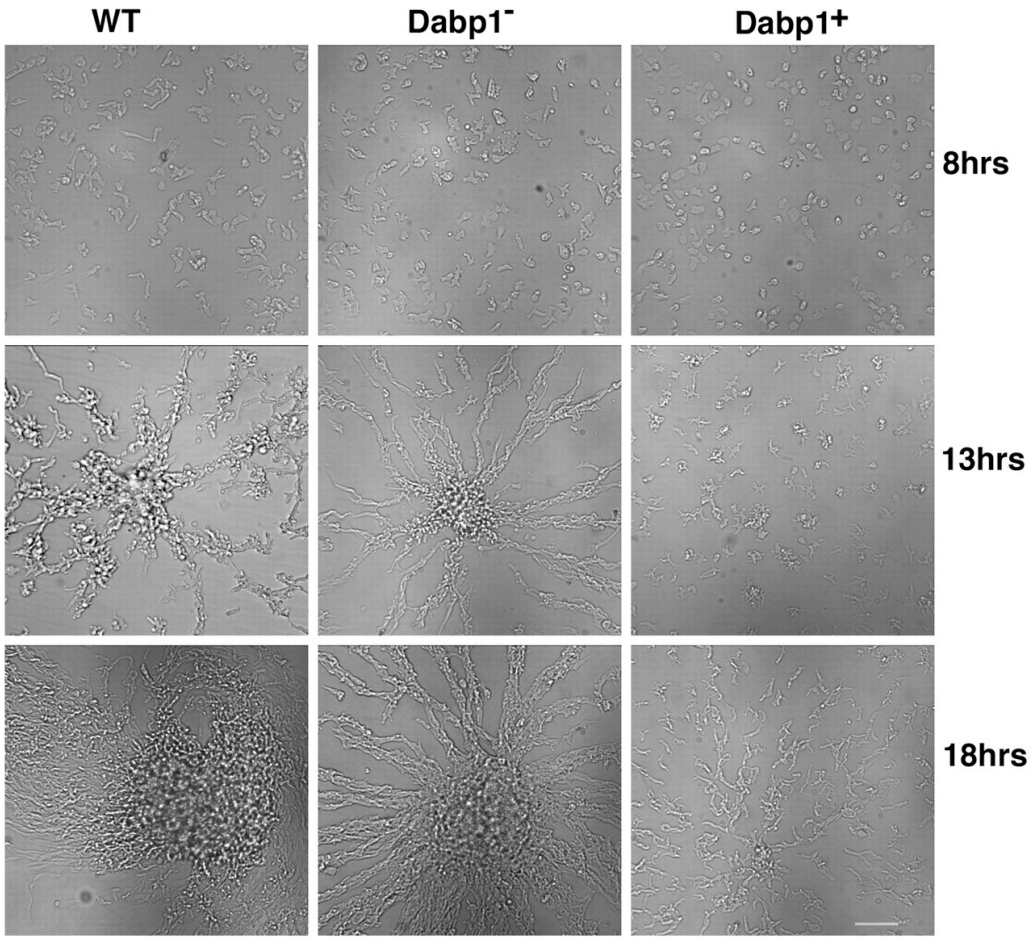

Figure 2.5 Overexpression of Dabp1 delays early development. Wildtype cells (WT), Dabp1 null cells (Dabp1$^-$) and cells overexpressing Dabp1 (Dabp1$^+$) were submerged under starvation buffer to induce chemotaxis, and photographed at various times as indicated. Bar, 100 μm.

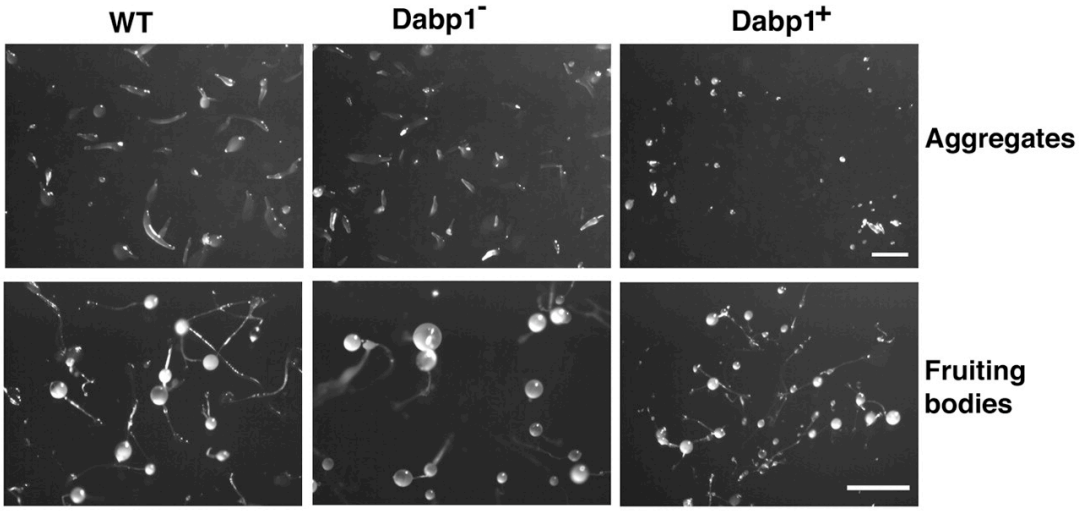

Figure 2.6 Overexpression of Dabp1 affects early development. Wildtype cells (WT), Dabp1 null cells (Dabp1$^-$) and cells overexpressing Dabp1 (Dabp1$^+$) were placed on nonnutrient agar plates. Images were taken of cells after the aggregation stage (Aggregates) and after the final fruiting body stage (Fruiting bodies). Bar, 100 μm.

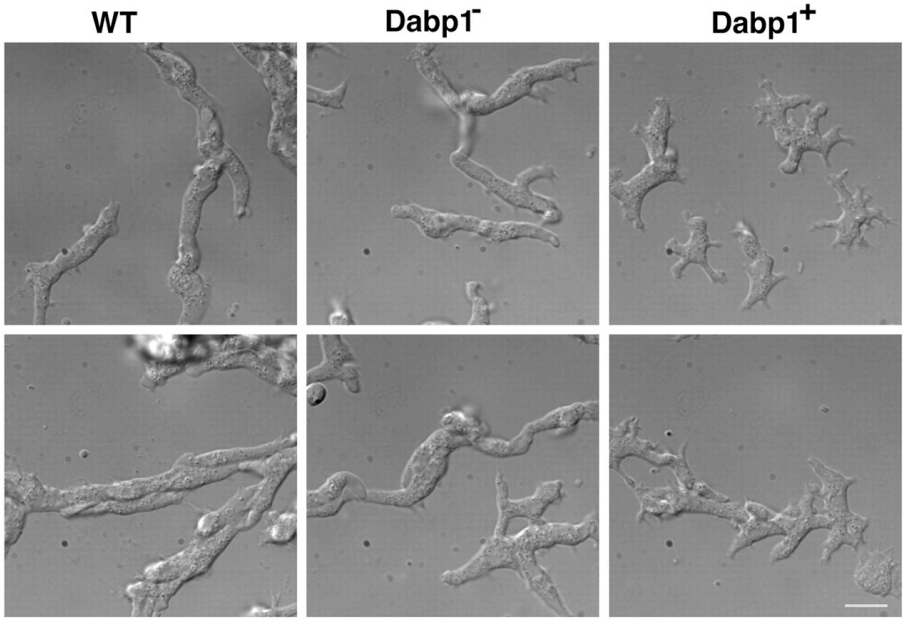

Figure 2.7 Overexpression of Dabp1 causes morphological changes during chemotaxis. Wildtype cells (WT), Dabp1 null cells (Dabp1$^-$) and cells overexpressing Dabp1 (Dabp1$^+$) were submerged under starvation buffer until they were actively streaming and photographed to study the morphology. Bar, 10 μm.

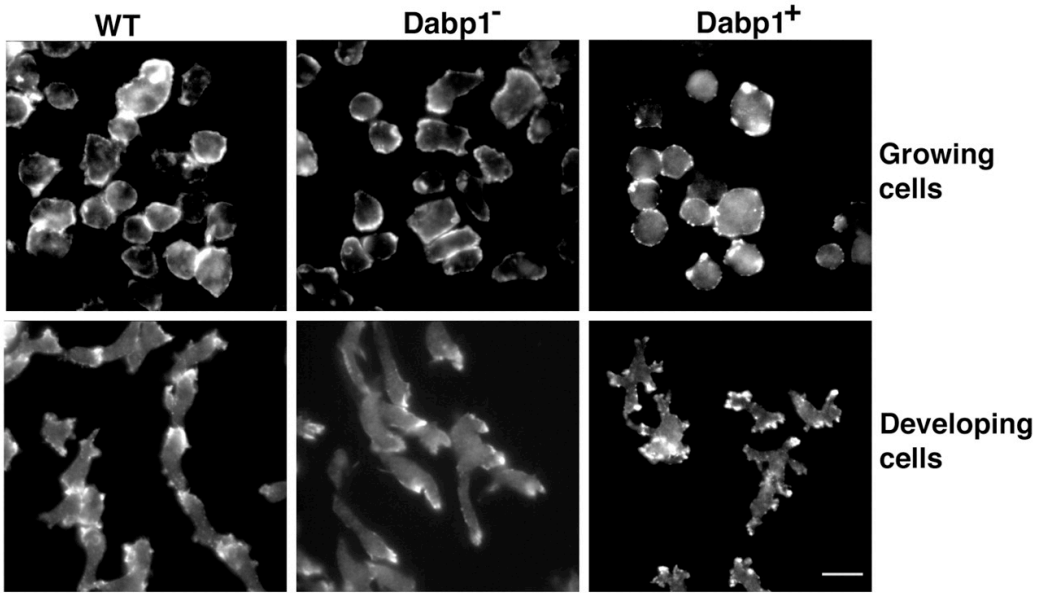

Figure 2.8 Actin distribution in supernumerary pseudopodia in Dabp1^{+} cells is normal. Wildtype cells (WT), Dabp1 null cells (Dabp1^{-}) and cells overexpressing Dabp1 (Dabp1^{+}) were grown in medium (Growing cells) or submerged under starvation buffer until they were actively streaming (Developing cells), and then fixed and stained with TXRED-labeled phalloidin to image the actin cytoskeleton. Bar, 10 μm.

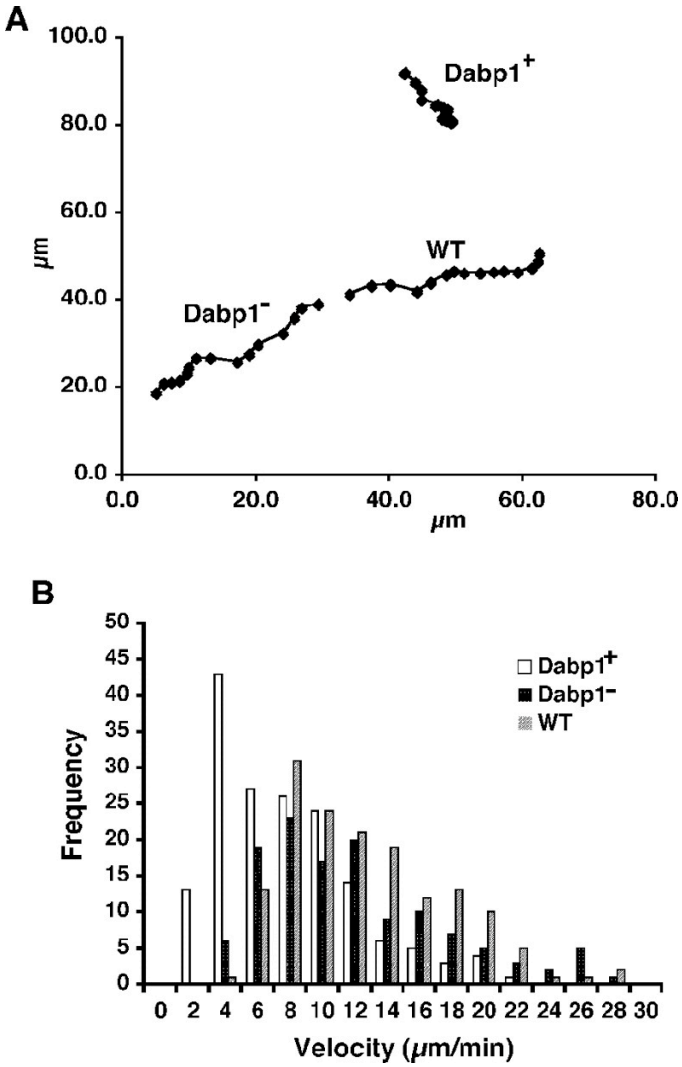

Figure 2.9 Dabp1 affects cell motility during chemotaxis in early development. (A) Wildtype cells (WT), Dabp1 null cells (Dabp1$^-$) and cells overexpressing Dabp1 (Dabp1$^+$) were submerged under starvation buffer until the cells were actively streaming. Cells were photographed every 12 seconds using DIC optics. Their positions at 15 time points are shown for a typical cell from each cell line. (B) Histogram showing velocities of wildtype cells (WT); Dabp1 null cells (Dabp1$^-$) and cells overexpressing Dabp1 (Dabp1$^+$) during chemotaxis in early development.

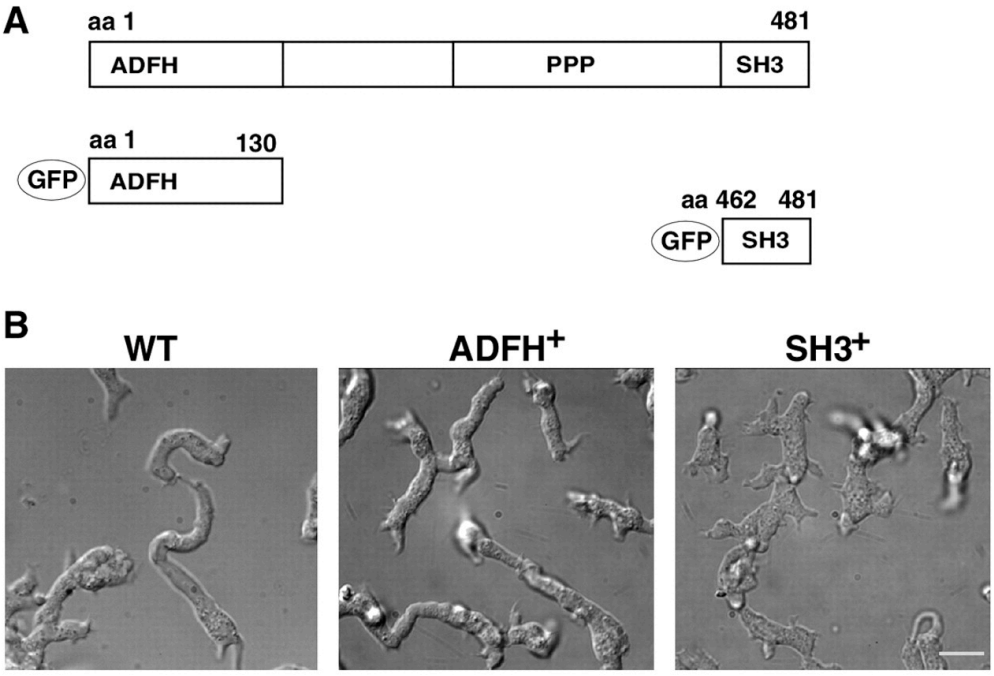

Figure 2.10 The SH3 domain is important for the function of Dabp1. (A) Diagram of constructs for ADFH and SH3 domain of Dabp1. (B) Morphology of wildtype cells (WT) and cells overexpressing the ADFH domain ($ADFH^+$) and the SH3 domain ($SH3^+$). (B) Cells were placed in the starvation buffer until they were actively streaming (about 9 hours) and then imaged under DIC optics. Bars, 10 µm.

Table 2.1 Dabp1 regulates the number of pseudopodia during chemotactic aggregation

Cell line	WT	Dabp1^{+}	Dabp1^{-}	SH3^{+}	ADFH^{+}
Percentage of cells having more than 2 cellular protrusions	7.5% ± 1%	89% ± 3.6%	26.5% ± 4%	87% ± 1.2%	18.4% ± 2%

Wildtype cells, Dabp1^{+} cells, SH3^{+} cells, ADFH^{+} cells and three independent Dabp1^{-} cell lines were used. Cells were submerged under starvation buffer until they were actively streaming, and then photographed using DIC microscopy. For each cell line, pseudopodia were quantified using cells imaged in three different experiments.

Chapter 3 MyoB requires Abp1 to regulate the actin structures during chemotaxis in *Dictyostelium*

INTRODUCTION

The actin cytoskeleton plays a key role in cell motility. Dynamic rearrangements of cortical actin drive the formation of F-actin filled projections such as lamellipodia, pseudopodia and filopodia. Regulation of these actin extensions are critical for cell motility and proteins involved in regulation of actin extensions must work cooperatively (Affolter and Weijer, 2005; Van Haastert and Devreotes, 2004).

When starved, *Dictyostelium* amoeba initiate a simple developmental program where they secrete extracellular cAMP that is used as a signal for chemotaxis. In response to the cAMP signal, the amoebae adopt a polarized morphology and stream together into aggregation centers. As they move, the *Dictyostelium* cells must reorganize their actin cortex to form a single dominant pseudopodium at the leading edge. To move persistently toward the extracellular signal, cells must also suppress the formation of lateral pseudopodia that would lead them away from the signal. Proteins important for controlling the number and formation of pseudopodia in *Dictyostelium* include the myosin I proteins, MyoA and MyoB; Myosin kinases such as MIHCK, PAKa and PAKc; and the actin-binding protein Dabp1 (Chung and Firtel, 1999; Chung et al., 2001; Lee et al., 2004; Wang and O'Halloran T, 2006; Wessels et al., 1991; Wessels et al., 1996).

Among these proteins, MyoB concentrates in the leading edge of the pseudopodium in chemotaxing cells (Fukui et al., 1989). The *Dictyostelium* myosin I, MyoB, plays an important role in regulating pseudopodia during chemotaxis. Deletion of MyoB increases

the number or lateral pseudopodia while overexpression of MyoB suppresses the formation of pseudopodia (Novak and Titus, 1997; Titus et al., 1993; Wessels et al., 1991; Wessels et al., 1996). These studies suggest that MyoB is a negative regulator of pseudopodia formation. Several proteins are known to regulate the activity of MyoB and work cooperatively with MyoB to control the formation of pseudopodia. For example, MyoB binds to the proline-rich region on the scaffold protein p116/Acan 125 via its SH3 domain. The Arp2/3 complex also interacts with p116/Acan 125 (Jung et al., 2001; Xu et al., 1997). Thus MyoB is linked to Arp2/3 complex at the leading edge, where actin polymerization is required for cell polarity and cell motility during chemotaxis. MyoB is also regulated by kinases: the phosphorylation of MyoB is essential for function and point mutations in the myosin I phosphorylation site of MyoB results in strong defects during chemotaxis (Falk et al., 2003; Gliksman et al., 2001; Wessels et al., 1996). Kinases that could regulate MyoB activity during chemotaxis are PAK members (*p*21-*a*ctivated protein *k*inases). PAKs are known to function in pseudopodia formation by phosphorylating and regulating the activity of Myosin II or Myosin I proteins (Chung and Firtel, 1999; Chung et al., 2001; Lee et al., 2004).

Similarly to MyoB, Dabp1 is enriched in the leading edge of the streaming cells and functions to limit pseudopodium number in chemotaxing *Dictyostelium* cells (Wang and O'Halloran T, 2006). At the amino-terminus Dabp1 contains an actin-biding domain whereas the carboxyl-terminus contains both poly-proline sequences and an SH3 domain that could bind regulators of pseudopodia formation (Wang and O'Halloran T, 2006).

Domains within Dabp1 suggest that it could serve as a scaffold within pseudopodium to couple proteins important for pseudopodium formation to the actin cytoskeleton.

Here we investigated a possible functional relationship between Dabp1 and MyoB in *Dictyostelium* cells. We found that Dabp1 is important for the spatial restriction of MyoB to the periphery of growing cells. Moreover, Dabp1$^-$/MyoB$^-$ cells exhibited deficiencies that were more severe than MyoB or Dabp1 single null mutants. During chemotaxis, these double mutants extended an increased number of pseudopodia that branched extensively. Dabp1$^-$ cells that overexpressed MyoB were phenotypically similar to Dabp1$^-$/MyoB$^-$ cells. In contrast, MyoB$^-$ cells that overexpressed Dabp1 were phenotypically similar to chemotaxing wildtype cells, and projected a dominant unbranched pseudopodium. We conclude that both Dabp1 and MyoB function in the regulation of actin extensions and that MyoB requires Dabp1 for proper localization to the cell cortex. We hypothesize that Dabp1 functions as a scaffold either to recruit MyoB or proteins that regulate MyoB at the cell cortex.

RESULTS

MyoB and Dabp1 co-localize at the cortex

Both Dabp1 and MyoB localize in the cell cortex and crown-like structures on the cell surface in growing cells (Novak et al., 1995; Wang and O'Halloran T, 2006). To compare the localization of these two proteins directly, we used affinity-purified anti-Dabp1 antibodies to immunostain cells expressing GFP-MyoB. Dabp1 was enriched at the cortex, particularly in lamellipodia, a structure known to contain dynamic actin. Like

Dabp1, MyoB was found in lamellipodia, but also outlined the cell periphery outside of lamilipodia. Comparison of the location of the two proteins showed that Dabp1 generally occupied a broader zone at the edge of cellular lamellipodia whereas MyoB was generally concentrated at the edge (**Fig. 3.1**).

Generation of Dabp1/MyoB double null mutants

The similarity in localization suggested that the MyoB and Dabp1 might function together in forming structures with active actin-like lamellipodia. To examine the relationship between the two proteins we generated a series of mutant cell lines. Using homologous recombination, we generated a Dabp1 null mutant (Dabp1$^-$) by deleting the Dabp1 gene from DH1 cells, the same parental line as MyoB null cells (MyoB$^-$). We also generated Dabp1 and MyoB double null mutants (Dabp1$^-$/MyoB$^-$) by deleting the Dabp1 gene from the MyoB$^-$ cell line. Western blots probed with MyoB antibodies or affinity-purified Dabp1 antibodies confirmed that MyoB and Dabp1 were not expressed in Dabp1$^-$/MyoB$^-$ mutants (**Fig. 3.2**). Initial characterization of the phenotype showed that both Dabp1$^-$ mutants and MyoB$^-$ mutants grew normally in suspension culture. Dabp1$^-$ /MyoB$^-$ mutants grew slower in suspension cultures with an increased percentage of multi-nucleated cells (data not shown). When the cells were put on nonnutrient agar plates, a surface on which *Dictyostelium* cells complete development to form multicellular fruiting bodies, Dabp1$^-$ cells, MyoB$^-$ cells, and Dabp1$^-$/MyoB$^-$ cells formed normal fruiting bodies and spores (data not shown).

Deletion of both Dabp1 and MyoB affect the organization of the actin cytoskeleton

While both Dabp1$^-$ and MyoB$^-$ mutants display multiple pseudopodia during chemotaxis in development, these mutants have a normal shape when growing in nutrient-rich media (Wang and O'Halloran T, 2006; Wessels et al., 1991; Wessels et al., 1996). To examine the possible effect of deleting both genes, we examined growing wildtype cells, MyoB$^-$ cells, Dabp1$^-$ cells and Dabp1$^-$/MyoB$^-$ cells after staining the actin cytoskeleton with TXRED-labeled phalloidin. Dabp1$^-$ cells and MyoB$^-$ cells showed an actin-rich cortex that was similar to that seen in wildtype cells. In contrast, in Dabp1$^-$/MyoB$^-$ cells, the cortical distribution of F-actin was disrupted by many small spikes filled with F-actin on the cell surface (**Fig. 3.3**). This phenotype indicated that the actin cytoskeleton was altered by the absence of both Dabp1 and MyoB proteins.

Dabp1 is important for the proper localization of MyoB

Since Dabp1 and MyoB partially co-localize, we examined the possibility that one protein influences the distribution of the other. We therefore tested the distribution of Dabp1 in MyoB$^-$ cells and the distribution of MyoB in Dabp1$^-$ cells. Triton x-100 was used to separate wildtype cells, Dabp1$^-$ cells and MyoB$^-$ cells into a triton-soluble fraction and a triton-insoluble cytoskeleton fraction. We then used western blots probed with anti-MyoB and anti-Dabp1 to assess the distribution of MyoB and Dabp1 in the fractions, respectively. Dabp1 fractionated into both the triton-soluble fraction and the triton-insoluble cytoskeleton. This fractionation pattern was similar in both wildtype cells and in MyoB$^-$ cells (**Fig. 3.4A**). In wildtype cells, MyoB was mostly found in the triton-soluble fraction with a small amount distributed into the triton-insoluble cytoskeleton.

However in Dabp1$^-$ cells, this fractionation pattern was disrupted: MyoB was absent from the triton-insoluble cytoskeleton (**Fig. 3.4A**). These results suggested that Dabp1 is important for the association of MyoB with the triton-insoluble cytoskeleton.

To examine whether Dabp1 functions in the proper localization of MyoB, fluorescence microscopy was used to assess the distribution of GFP-MyoB in wildtype cells and in Dabp1$^-$ cells. In wildtype cells, GFP-MyoB was concentrated tightly to the cell periphery. However, in Dabp1$^-$ cells, MyoB was more diffuse and occupied a much broader zone in the cell cortex (**Fig. 3.4B**). In contrast, the localization of Dabp1 was similar in MyoB null cells and in wild type cells, consistent with the results from triton cytoskeleton assays (data not shown).

Dabp1 and MyoB regulate the number and uniformity of pseudopodia in chemotaxing cells

The small actin-filled spikes observed in growing Dabp1$^-$/MyoB$^-$ cells suggested that these proteins could function together to maintain proper actin organization. To test whether these two proteins work together to regulate the actin structures during early development, we used Differential Interference Contrast (DIC) Microscopy to examine cells during chemotaxis. As reported previously, deletion of Dabp1 or MyoB resulted in increased pseudopodia formation (Wang and O'Halloran T, 2006; Wessels et al., 1996). During chemotaxis, most wild type cells (around 82%) formed two extensions from their cell body, a pseudopodium and a uropod, resulting in an elongated cell shape (**Table 3.1**). In contrast, ~40% of MyoB$^-$ cells and ~57% of Dabp1$^-$ cells projected more than two extensions from their cell body during chemotaxis (**Fig. 3.5A; Table 3.1**). Most of these

extensions were thick pseudopodia, although a small number of thin filopodia also projected from the cell body. In Dabp1$^-$/MyoB$^-$ cells, most (~90%) of cells exhibited more than two cell extensions. Many of the cell extensions branched extensively into fine projections at the leading edge. Moreover, the cell bodies of Dabp1$^-$ /MyoB$^-$ cells were covered with many thin cellular projections, filopodia (**Fig. 3.5A**; **Table 3.1**).

To examine the activity of these surface projections, we used DIC microscopy to image living wildtype cells, MyoB$^-$ cells, Dabp1$^-$ cells and Dabp1$^-$/MyoB$^-$ cells during chemotaxis. We focused on the emergence of smooth cellular extensions that were devoid of organelles as these are known to be filled with dynamic filamentous actin (Wessels et al., 1991). Wildtype cells extended projections that were smooth in the 30-second period of observation, and were stable and uniform in shape. Even when the projections branched, wildtype cells soon retracted back the branches and maintained a single projection (**Fig. 3.5B**). In contrast, chemotaxing MyoB$^-$ cells or Dabp1$^-$ cells extended smooth projections that were much less stable. The projections in these mutants soon formed up to 5 branches and these branches were maintained throughout the entire life of the smooth projection (**Fig. 3.5B**). Chemotaxing Dabp1$^-$/MyoB$^-$ cells were even less stable. These mutants extended up to 15 small branches from their smooth projection. Even after organelles entered the projections, the fine branches continued to extend from the cells, dramatically altering the shape of the cells (**Fig. 3.5B)**.

Dabp1 is important for the functions of MyoB in maintaining proper actin structures during chemotaxis

In the absence of MyoB, cells extend more lateral pseudopodia whereas overexpression of MyoB suppresses the formation of lateral pseudopodia (Novak and Titus, 1997; Wessels et al., 1991; Wessels et al., 1996). We therefore examined whether MyoB required Dabp1 to regulate these actin extensions by comparing the actin-filled extensions formed by wild type cells and Dabp1$^-$ cells expressing GFP-MyoB. Western blots stained with anti-MyoB antibodies confirmed similar expression levels of GFP-MyoB in the two cell lines (data not shown). As found previously, wildtype cells expressing GFP-MyoB failed to polarize and formed a round shape (Novak and Titus, 1997) (**Fig. 3.6A**). But when GFP-MyoB was expressed in Dabp1$^-$ cells, cells polarized and formed multiple pseudopodia that branched extensively (**Fig. 3.6A; Table 3.1)**. DIC imaging revealed that branches at the leading edge of the pseudopodia in Dabp1$^-$ cells overexpressing MyoB were stable throughout the life of the pseudopodia (**Fig. 3.6B**). This phenotype differed from the phenotype of Dabp1$^-$ cell, and closely resembled the increased deficiency exhibited by Dabp1$^-$/MyoB$^-$ cells. These results suggested that Dabp1 was required for the proper function of MyoB in regulation of pseudopodia.

Dabp1 shares redundant functions with MyoB in controlling pseudopodia formation during chemotaxis

The preceding experiments established a requirement of Dabp1 for MyoB function. To examine the reciprocal, a possible requirement of MyoB for Dabp1 function, we expressed GFP-Dabp1 in wildtype and MyoB$^-$ cells. Western blot analysis confirmed that equivalent amounts of the protein were expressed in each cell line (data not shown). In contrast with Ax2 cells, where overexpression of GFP-Dabp1 yielded a dominant

negative phenotype of multiple pseudopodia (Wang and O'Halloran T, 2006), overexpression of GFP-Dabp1 in the DH1 cell line had little effect. ~40% of MyoB$^-$ cells had more than two cellular projections (**Fig. 3.7A; Table 3.1**). Surprisingly, overexpression of GFP-Dabp1 in MyoB$^-$ cells reduced the number of cells displaying a multi-pseudopodia phenotype, and only ~ 18% of cells projected more than two extensions, a fraction equivalent to that observed in wildtype cells. This indicated that overexpression of Dabp1 rescued the multi-pseudopodia defect of MyoB$^-$ cells.

The Dabp1 protein has an ADFH domain at its amino-terminal domain and an SH3 domain at its carboxyl-terminus. The ADFH domain is an actin-binding domain while the SH3 domain is a linker to other proline-rich containing proteins or other SH3 domain containing proteins. When MyoB$^-$ cells only overexpressed the ADFH domain, they were not rescued for the defects in pseudopodia formation, and 42% of the cells extended more than two protrusions (**Fig. 3.7B; Table 3.1**). But when the SH3 domain was overexpressed in MyoB$^-$ cells, the percentage of cells extending more than two cellular projections dropped to ~16%, close to the fraction observed for wild type cells (**Fig. 3.7B; Table 3.1**).

DISCUSSION

During chemotaxis, cells reorganize their actin cytoskeleton to extend pseudopodia toward extracellular cues. In order for cells to move efficiently, the timing, position, and number of these actin-filled protrusions must be tightly controlled. To move persistently in the right direction, cells extend a single dominant pseudopodium that forms a leading

edge directed toward the signal, and limit lateral pseudopodia oriented away from the signal. Some of the cellular regulators for the actin cytoskeleton that control pseudopodia formation during chemotaxis have been identified by the study of phenotypes of mutant *Dictyostelium* cells. MyoB and MyoA null mutants project extra pseudopodia that impair cell motility during chemotactic aggregation (Wessels et al., 1991; Wessels et al., 1996). *Dictyostelium* cells that overexpress Dabp1 also project pseudopodia that perturb the efficient movement of cells during aggregation (Wang and O'Halloran T, 2006). Here we show that Dabp1 and MyoB work cooperatively in regulating actin extensions during chemotaxis. Dabp1 is required for the proper localization of MyoB as Dabp1 null cells show an expanded zone of MyoB at the cortex. Overexpression of Dabp1 can rescue the defects association with the deletion of MyoB. However, deletion of both Dabp1 and MyoB results in an increased number of pseudopodia, and also alters the uniformity of pseudopodia resulting in extensive branches at the leading edge.

Dabp1 regulates the localization of MyoB

MyoB is one of the three long-tailed Myosin I proteins containing three domains (TH1, TH2 and TH3) in the tail region (de la Roche and Cote, 2001). TH1 domains are rich in basic amino acids, which can bind anionic phospholipids, and function to anchor MyoB to the membrane periphery (Doberstein and Pollard, 1992; Senda and Titus, 2000). In growing Dabp1 null cells, we found that MyoB lost its membrane localization and localized to an expanded zone within the cell cortex. Our results suggest that the TH1 domain of MyoB is not the sole determinant for localization on the plasma membrane and that Dabp1 also regulates the localization of MyoB. Previous studies suggest that proteins

regulating the specific binding of MyoB to the plasma membrane may exist both in the cytosol and on the plasma membrane (Senda and Titus, 2000). Dabp1 might be this type of regulator.

Two regions on MyoB could serve as potential binding sites for Dabp1. First, the TH2 domain (GPA domain) on the tail has a high content of glycine, proline and either alanine or glutamine residues (de la Roche and Cote, 2001). GPA domains strongly resemble the proline-rich domains (PRD's) of WASP/SCAR and formins, and can serve as substrates for SH3-containing proteins (Evangelista et al., 2002; Mullins, 2000). Since the GPA domain of another long-tailed myosin I, MyoK, has been reported to bind the SH3 domain of Dabp1 (Soldati, 2003), the GPA domain of MyoB has potential for binding the SH3 domain of Dabp1. Second, direct binding between Dabp1 and MyoB could be mediated by the TH3 domain (an SH3 domain) on the tail of MyoB interacting with the proline-rich region on the carboxyl-terminal region of Dabp1. Alternatively, rather than binding MyoB directly, Dabp1 could interact with regulators of MyoB that are important for its localization to the cell periphery. By associating with the actin cytoskeleton via the ADFH domain at the amino-terminus, and binding MyoB or its regulators with the GPA or the polyproline domain at the carboxyl-terminus, Dabp1 could serve as a scaffold to recruit MyoB to the actin cortex within the cell.

The SH3 domain of Dabp1 functions in the regulation of pseudopodia formation

When Dabp1 was overexpressed in MyoB null cells, the defects caused by loss of MyoB were rescued. Chemotaxing cells recovered the ability to suppress additional pseudopodia and behaved as wildtype cells. This rescue can be attributed to the SH3

domain since overexpression of the SH3 domain of Dabp1 also rescued the multi-pseudopodia defect caused by deletion of MyoB. Conceivably the overexpression of Dabp1 could recruit lower affinity proteins that can substitute for MyoB. These could include either other myosin I proteins such as MyoA, a myosin 1 already known to share redundant functions with MyoB, or regulators of the Myosin I family (Falk et al., 2003). Potential regulators for myosin I include members of the PAK (*p*21-*a*ctivated *k*inase) family. PAK kinases phosphorylate and activate Myosin I proteins. The amino-terminal regulatory domain of several PAKs contain proline-rich sequences that can mediate interactions with SH3 domain-containing proteins (Bagrodia and Cerione, 1999; Brzeska et al., 1997; de la Roche and Cote, 2001; Wu et al., 1996). Thus the overexpression of Dabp1 or its SH3 domain could bind the PAK kinases and increase the activation of Myosin I proteins such as MyoA. Since MyoA plays redundant functions with MyoB in pseudopodia formation, production of more activated MyoA could potentially rescue the defects caused by deletion of MyoB (Titus et al., 1993; Wessels et al., 1996). Indeed in initial studies, I found that MyoA mutants (37.4% $\pm$ 1.7) displayed an increased number of pseudopodia, and this defect could be rescued by overexpression of Dabp1 in the MyoA null cells (data not shown).

MyoB requires Dabp1 to regulate the number and uniformity of pseudopodia during chemotaxis

Dabp1/MyoB double null cells exhibited an increased number of chemotaxing cells with multiple pseudopodia. In addition to the increased number of pseudopodia in these mutants, we also found that the pseudopodia branched extensively at the leading edge, a

more severe defect than seen in MyoB or Dabp1 single mutants. The increased defect in the double mutant suggests that Dabp1 and MyoB work cooperatively in regulating the formation of smooth, unbranched pseudopodia.

To shape a smooth and uniform pseudopodium, MyoB could generate contractile force to retract additional F-actin filled projections caused by dynamic actin polymerization (Dai et al., 1999). Dabp1 and MyoB are both concentrated at the leading edge (Fukui et al., 1989; Wang and O'Halloran T, 2006). We found that expression levels of MyoB are sensitive to Dabp1. Without Dabp1, overexpression of MyoB caused cells to extend increased number of pseudopodia. These pseudopodia were so unstable that they broke into numerous branches, a phenotype similar to Dabp1/MyoB double null mutants. Interestingly, Dabp1 null cells that express endogenous MyoB, were capable of regulating the integrity of pseudopodia although they project an increased number of lateral pseudopodia. One possible reason is that deletion of Dabp1 could result in a weakened cell cortex. Consequently either too little or two much MyoB in Dabp1 null cells could exacerbate this defect, causing the weak cortex to project actin-filled spikes.

We propose that Dabp1 and MyoB work as a team to regulate actin extensions during chemotaxis (**Fig. 3.8**). Dabp1 acts as a scaffold to recruit MyoB and/or regulators of MyoB to sites of dynamic actin polymerization. MyoB and Dabp1 restrict the projection of lateral pseudopodia and also function at the leading edge to maintain a uniform leading edge. Together MyoB and Dabp1 regulate the position, number, and integrity of actin extensions during chemotaxis.

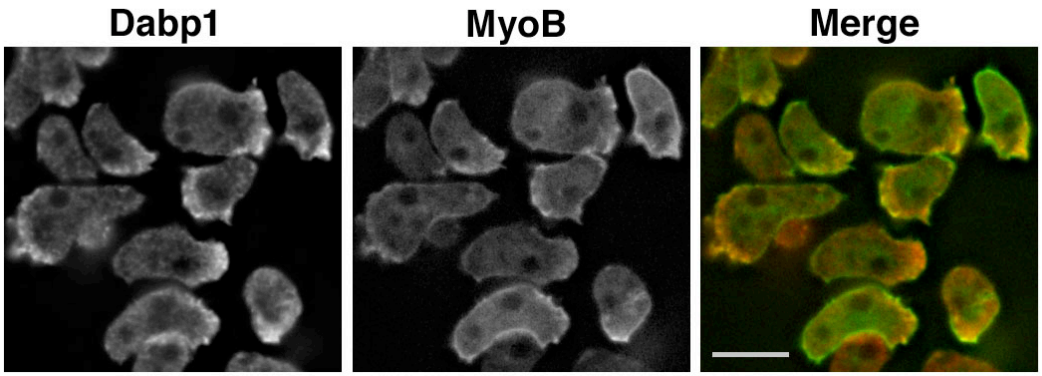

Figure 3.1 Dabp1 and MyoB co-localize at the cortex. Wildtype cells expressing GFP-MyoB were fixed and stained with affinity-purified anti-Dabp1 antibodies followed by TXRED labeled secondary antibodies. The merged image shows that Dabp1 occupies a broader zone in some areas of the cortex whereas MyoB is more tightly associated with the cell periphery. (Green: GFP-MyoB; Red: Dabp1). Bar, 10 μm.

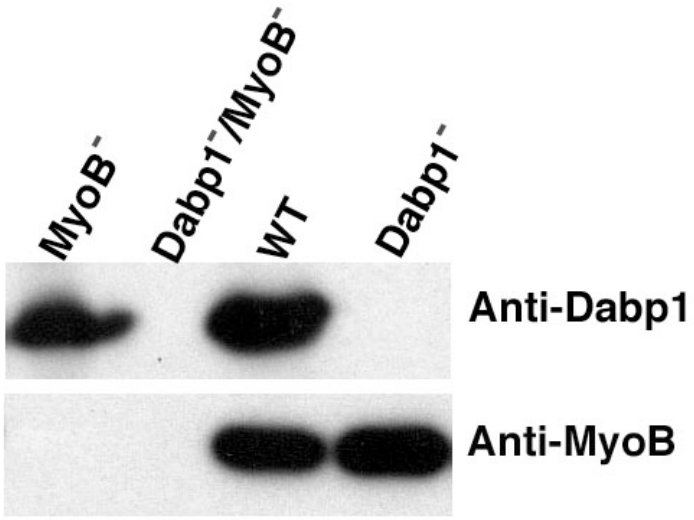

Figure 3.2 Generation of Dabp1$^-$/MyoB$^-$ cells. Cell lysates from 1x10^6 of wildtype cells, Dabp1$^-$ cells, MyoB$^-$ cells and Dabp1$^-$/MyoB$^-$ cells were loaded in each lane. Westerns were stained with affinity-purified anti-Dabp1 antibodies or with anti-MyoB antibodies.

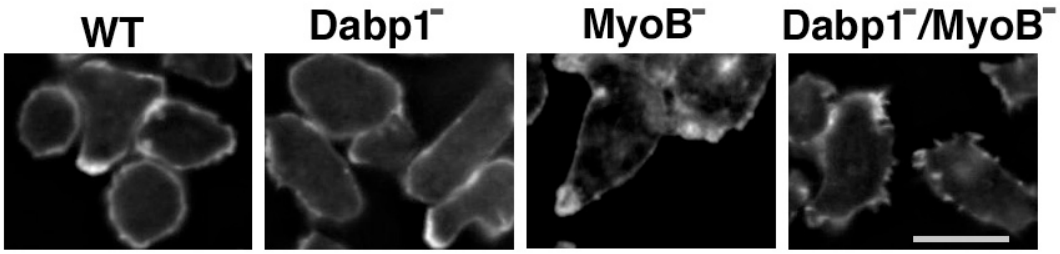

Figure 3.3 The actin cytoskeleton is altered in growing Dabp1$^-$/MyoB$^-$ cells. Wildtype cells, Dabp1$^-$ cells, MyoB$^-$ cells and Dabp1$^-$/MyoB$^-$ cells growing in rich media were fixed and stained with TXRED-labeled phalloidin to observe the distribution of F-actin. Bar, 10 μm.

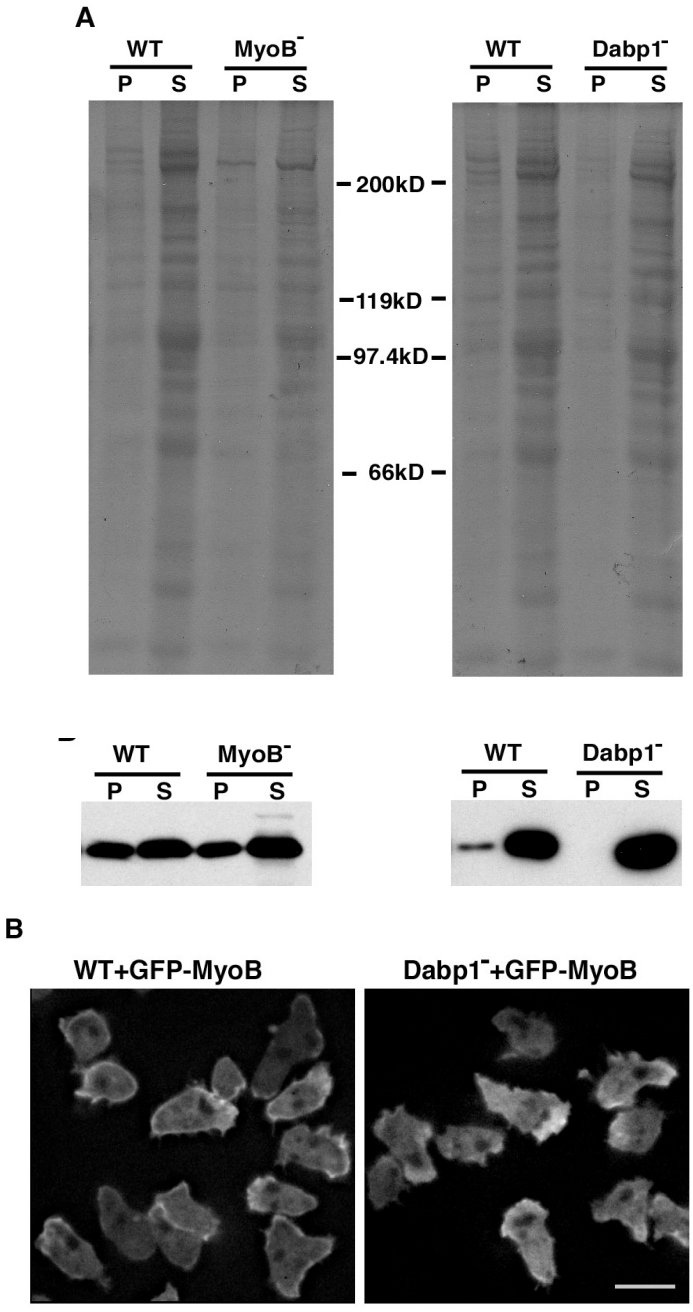

Figure 3.4 Dabp1 is important for the proper localization of MyoB. **(A)** The distribution of MyoB into the Triton-insoluble fraction is Dabp1 dependent. Wildtype cells, Dabp1$^-$ cells and MyoB$^-$ cells were pelleted and resuspended into Triton x-100 lysis buffer. The supernatants and the triton-insoluble pellets from each cell line were loaded equally on SDS gels and stained with Coomassie Blue or analyzed in western blots stained with anti-Dabp1 antibodies or with anti-MyoB antibodies **(B)** Dabp1 is required for the localization of MyoB to the cell periphery. Wildtype (WT) or Dabp1$^-$ cells that expressed GFP-MyoB were examined by fluorescence microscopy.

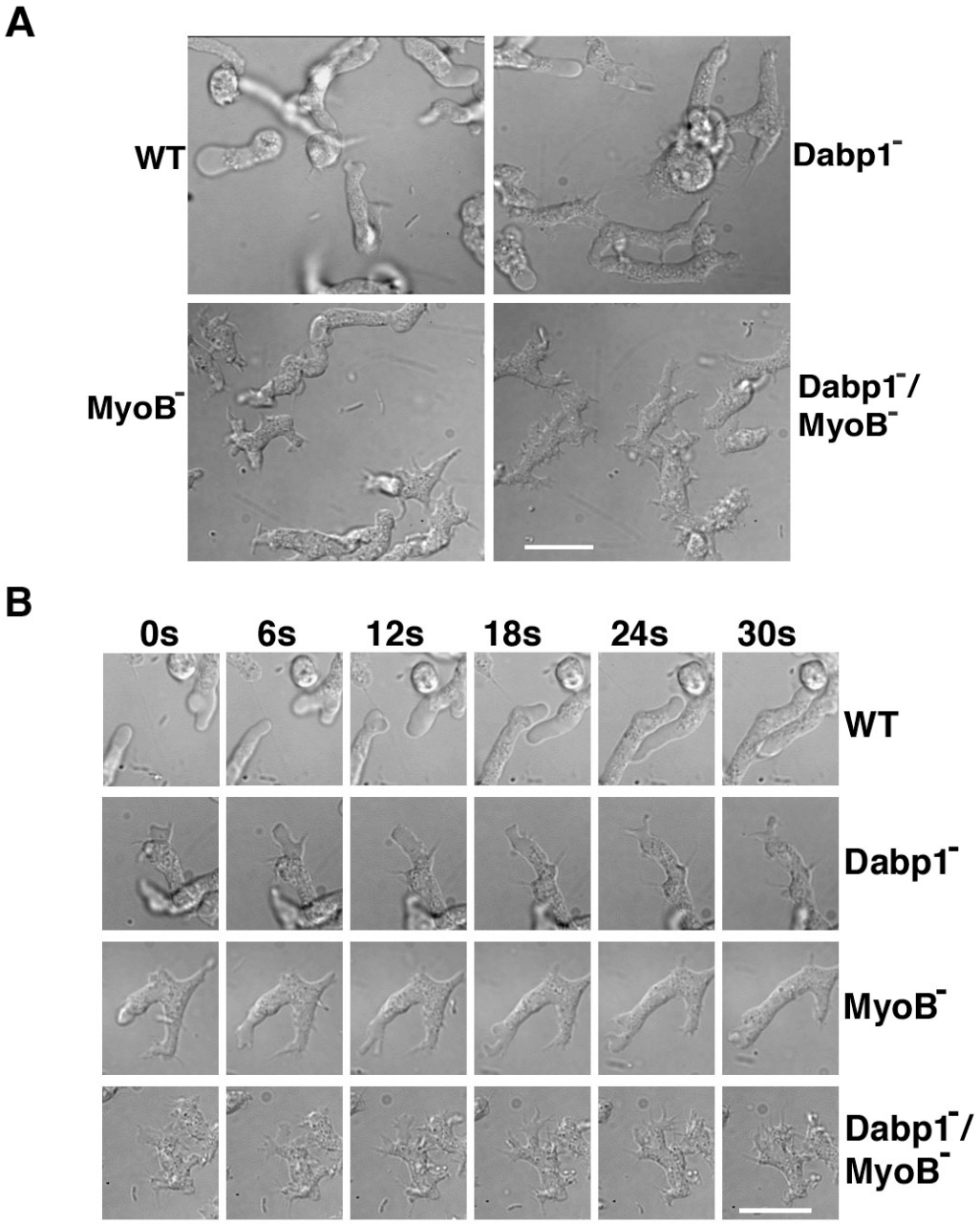

Figure 3.5 Dabp1 and MyoB regulate the number and uniformity of actin extensions during chemotaxis. **(A)** Dabp1$^-$/MyoB$^-$ cells project more pseudopodia with extensive branches during chemotaxis. Wildtype cells, Dabp1$^-$ cells, MyoB$^-$ cells and Dabp1$^-$ /MyoB$^-$ cells were submerged under starvation buffer until they were actively streaming and then imaged using DIC optics. Bar, 10 μm. **(B)** Time course of pseudopodia extension in wildtype cells, Dabp1$^-$ cells, MyoB$^-$ cells and Dabp1$^-$/MyoB$^-$ cells. Cell were starved until they were actively streaming, and smooth extensions were imaged every 6 seconds. Bar, 10 μm.

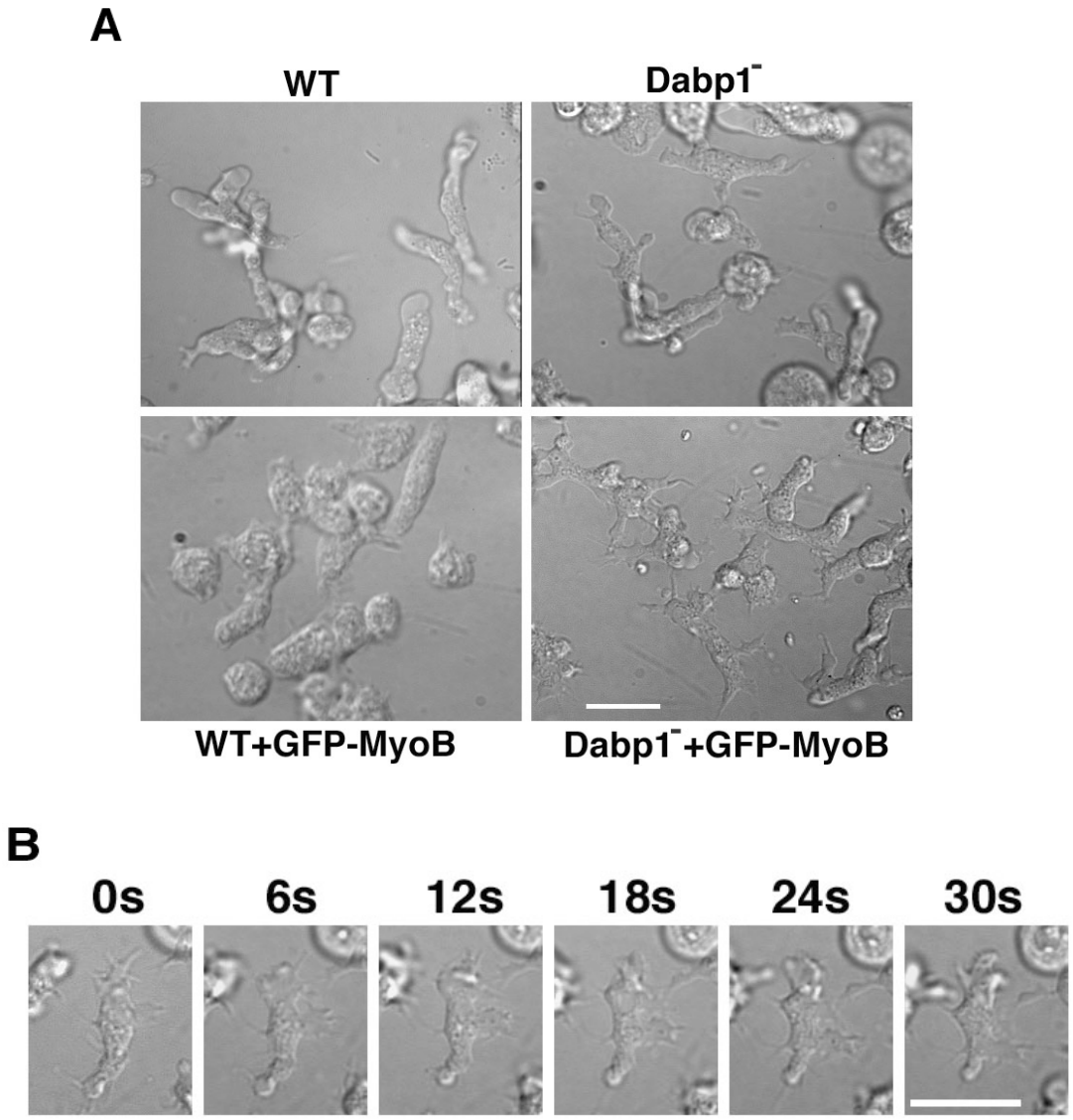

Figure 3.6 Dabp1 is important for the functions of MyoB in maintaining proper actin structures during chemotaxis. **(A)** Expression of MyoB in Dabp1$^-$ cells increases the number of pseudopodia with many small branches during chemotaxis. Wildtype cells, Dabp1$^-$ cells, wildtype cells expressing MyoB and Dabp1$^-$ cells expressing MyoB were placed in starvation buffer and imaged with DIC optics after cells were actively streaming. Bar, 10 μm. **(B)** MyoB requires Dabp1 to function in regulating the uniform shape of actin extensions during chemotaxis. Actively streaming cells from Dabp1$^-$ cells expressing MyoB were imaged with DIC optics every 6 seconds. Bar, 10 μm.

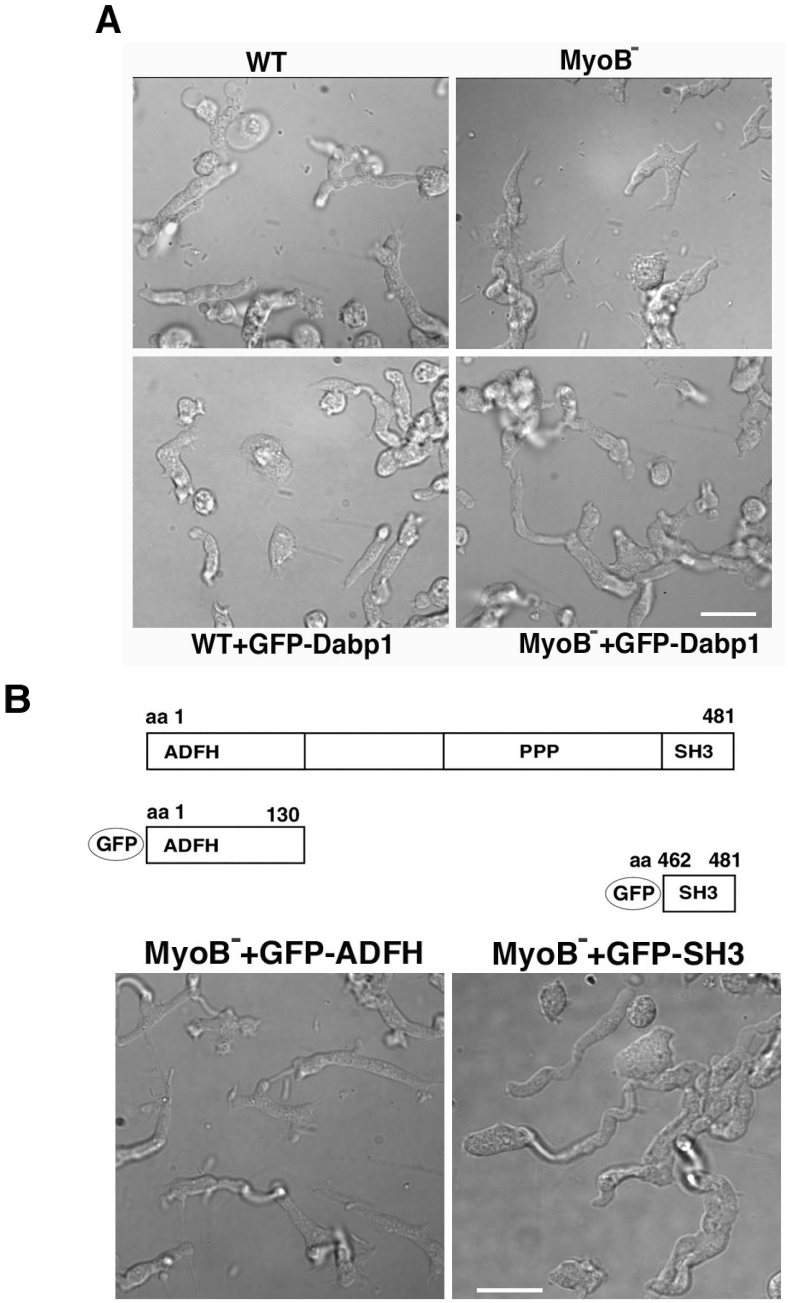

Figure 3.7 Dabp1 shares redundant functions with MyoB in controlling pseudopodia formation during chemotaxis. **(A)** Overexpression of Dabp1 suppresses the formation of supernumerary pseudopodia in MyoB null cells during chemotaxis. Wildtype cells, MyoB$^-$ cells and MyoB$^-$ cells expressing Dabp1 were submerged under starvation buffer and imaged while they were streaming under DIC optics. Bar, 10 µm. **(B)** The SH3 domain of Dabp1 is important for the suppression of lateral pseudopodia formation during chemotaxis. MyoB$^-$ cells expressing SH3 and MyoB$^-$ cells expressing ADFH were imaged after they were steaming in starvation buffer. Bar, 10 µm.

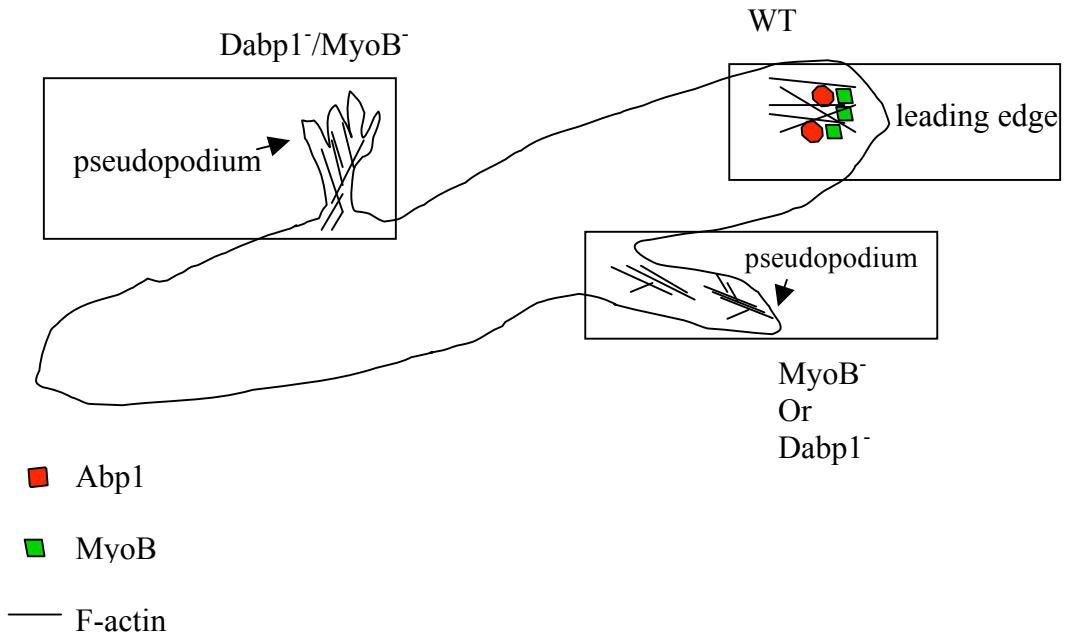

Figure 3.8 Working model for Dabp1 and MyoB functions during chemotaxis in *Dictyostelium.* In this model, MyoB works as an actin motor protein to reel in F-actin-filled protrusions. Dabp1 works as a scaffold to recruit MyoB and/or regulators of MyoB to sites of dynamic actin polymerization. In wildtype cells, both MyoB and Dabp1 localize at the leading edge where MyoB and Dabp1 regulate the uniformity of the leading edge. In MyoB$^-$ cells, cells failed to pull back the extensions caused by actin polymerization, which results in increased number of lateral pseudopodia. In Dabp1$^-$ cells, MyoB is not localized properly to the cell periphery and polymerized F-actin extends from the cell resulting in an increased number of pseudopodia. In Dabp1$^-$/MyoB$^-$ cells, the actin cortex is particularly deficient and the pseudopodia break into many small branches.

Table 3.1 Dabp1 and MyoB regulate pseudopodia formation during chemotaxis

Cell lines	Percentage of cells having more than two protrusions
WT	17.6% ± 2.1%
MyoB$^-$ cells	40.1% ± 3.3%
Dabp1$^-$ cells	57% ± 3.3%
Dabp1$^-$/MyoB$^-$ cells	90% ± 2.1%
WT expressing Dabp1	13% ± 1.9%
MyoB$^-$ cells expressing Dabp1	17.5% ± 2%
MyoB$^-$ cells expressing ADFH	42% ± 2.2%
MyoB$^-$ cells expressing SH3	16% ± 2.5%
WT expressing MyoB	n/a (most cells round up)
Dabp1$^-$ cells expressing MyoB	89.5% ± 2.1%

Cells from each cell lines in the table were placed into starvation buffer and imaged were taken until they were actively streaming. For each cell line, around 200 cells were selected from three independent experiments. Cells with more than two protrusions were counted. A standard deviation was calculated for each cell line.

Chapter 4 Abp1 is involved in formation of early endosomes in the *Dictyostelium* endocytic pathway

INTRODUCTION

In *Dictyostelium*, endocytosis is the process in which cells internalize nutrients. Phagocytosis and pinocytosis are two types of endocytic pathways involved in internalization of energy-rich particles and fluid, respectively. During the endocytic pathway, cargo is first rapidly engulfed by F-actin filled membrane extensions, internalized, and then delivered to early endosomes. The early endosomes go through a series of changes, including removal of the actin coat, acidification and transition into post-lysosomes. While the endosomes travel through the cells, their cargo is digested by digestive enzymes. Finally, the transport of cargo is linked to exocytosis to release undigested remnants and to recycle the membrane components (Cardelli, 2001; Maniak, 2001; Maniak, 2002; Maniak, 2003).

The endocytic pathway is a very complicated process involving the cooperation of many proteins. At early stages of endocytosis, the actin cytoskeleton must be reorganized to form membrane extensions to engulf cargo. The resulting endocytic vacuoles, initially coated with actin, are called early endosomes, and eventually deliver the cargo to lysosomes. The formation of early endosomes is very rapid, around 1 min (Maniak, 2003). Many proteins now are known to function in this rapid remodeling of the actin cytoskeleton. Among these proteins, the Arp2/3 complex, along with its activators such as the WASP/ SCAR proteins, controls the polymerization of F-actin (Ibarra et al., 2005;

Lee et al., 2001). Other actin-associated proteins are thought to fine-tune and regulate the process and to increase the efficiency of actin-mediated pinocytosis and phagocytosis (Maniak et al., 1995; Rybakin and Clemen, 2005). One regulator is the protein coronin that associates with actin and accumulates at endocytic cups (Maniak et al., 1995). Another class of regulators are the myosin I proteins which play roles in establishing and maintaining cortical tension (de la Roche and Cote, 2001; Soldati, 2003). In the Myosin I family, MyoA, B and C function in controlling the formation of F-actin filled membrane projections important in pinocytosis while both MyoB and MyoK are involved in phagocytosis (Novak et al., 1995; Schwarz et al., 2000). These regulators can be linked directly to the actin polymerization machinery. For example, MyoB and MyoC function to recruit the actin regulator Arp2/3 complex through the adaptor proteins CARMIL and Acan 125 (Jung et al., 2001; Xu et al., 1997). The SH3 domain of MyoB and MyoC are used to bind CARMIL and Acan 125 and thus are important crosslinkers within the meshwork of the actin cytoskeleton (Jung et al., 2001).

Dabp1 is known to have a SH3 domain at the C-terminus and a proline–rich region amino-terminal to the SH3 domain. One of the Myosin I proteins, MyoK binds Dabp1 through its GPR domain, a stretch of amino acids rich in glycine, proline and arginine (Schwarz et al., 2000; Soldati, 2003). So Dabp1 might function in endocytosis via MyoK. It is also possible that MyoB or MyoC binds to Dapb1 through their SH3 domains and thus link Dabp1 to endocytic pathway. Here I report on my studies of the contribution of Dabp1 to endocytosis. I found that (1) Dabp1 accumulated in crowns and endocytic cups, and co-localized with actin at these sites; (2) Dabp1 was associated with early

endosomes; (3) Deletion of Dabp1 had no effect on either pinocytosis of fluid-phase markers or on phagocytosis of bacteria. These results suggest that Dabp1 could play a non-essential role in the early stage of endocytosis.

RESULTS

Dabp1 accumulated in crowns and endocytic cups

To determine the location of Abp1 in *Dictyostelium,* we fixed growing cells and stained these with affinity-purified anti-Dabp1 antibodies. Dabp1 was found to accumulate in crowns, endocytic cups formed by extensions of the plasma membrane, and vacuolar structures inside the cells (**Fig. 4.1A, arrows**). F-actin is also known to be enriched in these structures. To examine the relationship between Dabp1 and F-actin at these locations, fixed cells were stained simultaneously with TEXAS-Red labeled phalloidin to label F-actin and with antibodies against Dabp1. Extensive co-localization between Dabp1 and actin was detected in these cells (**Fig. 4.1B**).

Association of Dabp1 in formation of early endosomes

The localization of Dabp1 on endocytic cups suggests that Dabp1 might function in the early stage of endocytic pathway. To investigate this possibility, I examined the localization of Dabp1 during phagocytosis. Since the original GFP-Dabp1 plasmid was expressed at high levels that generated a dominant negative phenotype, I generated an integrating plasmid for expression of GFP-Dabp1. Cells expressing the integrating plasmid analyzed by western blots showed that these cells expressed lower levels of GFP-Dabp1 than the original multi-copy expression plasmid. Moreover these cells did

not display a dominant negative phenotype (data not shown). Imaging cells with this integrating GFP-Dabp1 plasmid by fluorescence microscopy revealed that GFP-Dabp1 had localization similar to that in wild type cells immnuostained with anti-Dabp1 antibodies, which suggested that the images of GFP-Dabp1 in these cells reflected the normal localization of GFP-Dabp1 (**Fig. 4.2A**).

To examine the dynamic localization of Dabp1 during early stages of the endocytic pathway, living cells expressing the integrating GFP-Dabp1 plasmid were imaged during phagocytosis. Cells were incubated with heat-killed yeast and examined under fluorescence microscopy. When cells initially encountered yeast, they extended their plasma membrane in order to form cup-like structures to engulf the yeast. Dabp1 first accumulated at the leading edge of the endocytic cups and then moved to the bottom of the cup until the whole front edge of the cup, containing the engulfed yeast, was labeled with Dabp1. Dabp1 stayed on these structures until engulfment was finished and early endosomes formed, and then disassociated from the early endosomes (**Fig. 4.2B, arrow head**). The process took about 1 minute. During endocytosis, early endosome needs to shed its actin coat (Maniak, 2003). It is not clear that whether Dabp1 was shed with the actin coat or disassociated earlier than the actin coat.

Dabp1 null cells are normal in pinocytosis and phagocytosis

To determine whether Dabp1$^-$ cells had defects in pinocytosis, the uptake of FITC-dextran by wildtype cells and Dabp1$^-$ cells was analyzed. During the two-hour time course, the uptake of FITC-dextran by Dabp1$^-$ cells was measured at 6 different time points. Dabp1$^-$ cells had almost the same intake of FITC-dextran as wild type AX2 cells

for the first 30 minutes. At 120 minutes, both cell lines almost reached their maximum intake and the internalization of Dabp1$^-$ cells was approximately the same as wildtype cells (**Fig. 4.3A**). This data suggested that Dabp1 was not necessary for pinocytosis.

To determine whether Dabp1 is required for phagocytosis of *Dictyostelium* cells, equivalent numbers of Dabp1$^-$ cells and wildtype cells were each spotted on agar plates covered with an *E.coli* lawn. Using the *E.coli* as a food source, the cells cleared circles on the lawn. Thus the size of the growing circles was a measure of the ability of cells to phagocytose and internalize particles. After 5 days incubation, the size of growing circles on both wildtype cell plates and Dabp1$^-$ cell plates was measured. We found that both cell lines cleared a circle of approximately the same size (**Fig. 4.3B**). Data was also collected after 12 days incubation and no difference was noticed between Dabp1$^-$ cells and wildtype cells (data not shown). Therefore, Dabp1$^-$ cells had not defects on phagocytosis.

DISCUSSION

Dabp1 localizes to crowns, endocytic cups or early endosomes in growing cells and co-localizes extensively with actin at these sites. These results indicated that Dabp1 is associated with the formation of endosomes during the endocytic pathway. However, deletion of Dabp1 did not impair the ability of cells to internalize and transport cargo, which suggested that some other proteins play redundant functions with Dabp1. During the early stage of endocytosis, an actin coat is required for the formation of early endosomes. Therefore, proteins involved in actin polymerization are important for this activity. MyoB or MyoC is linked to actin assembly through P116/Acan 125 (Jung et al.,

2001; Xu et al., 1997). Moreover, Myosin I proteins are important to generate contractile force for the dissociation of early endosomes form membrane (de la Roche and Cote, 2001; Soldati, 2003). If Dabp1 acts as a scaffold to recruit MyoB, as proposed, this might explain why Dabp1 is found to localize in endocytic cups and early endosomes. Another explanation is that Dabp1 is associated with actin-based phagocytosis via its binding with MyoK (Schwarz et al., 2000; Soldati, 2003). It is also possible that some unknown proteins link Dabp1 to the early stage of endocytosis. In yeast, a screen for ligands of the SH3 domain of Abp1 identified six ligands (Fazi et al., 2002). Dabp1 might also bind to some proteins through its SH3 domain and then gets involved into endocytosis. It is also known that yeast Abp1 is a weak activator of Arp2/3 complex since it contains the two DDW motifs (Goode et al., 2001). Yeast Abp1 can be directly associated with actin assembly during the nucleation of actin filaments. The amino acid sequence of Dabp1 contains a single DDW motif in a position similar to yeast Abp1. Possibly, Dabp1 is also a weak activator of Arp2/3 complex. In support of this, Dabp1 always localizes to the regions of dynamic and active actin. Dabp1 preferentially associates with F-actin in the cells (Wang and O'Halloran T, 2006). Taken together, Dabp1 could play a role in the formation of actin coat on early endosomes by acting as an activator for Arp2/3 complex.

While associated with early endosomes, Dabp1 is not essential for endosome function. This result differs from the function of mammalian Abp1 in receptor-mediated endocytosis. In mammalian cells, Abp1 binds to dynamin via its SH3 domain. Overexpression of Abp1 dramatically reduced endocytosis in mammalian cells (Kessels

et al., 2001). To further explore how Dabp1 functions in endocytosis, studies of Dabp1$^-$/MyoB$^-$ cells or Dabp1$^-$ /MyoK$^-$ cells might help to answer the questions.

Collectively, the studies of Dabp1 in endocytosis point to a new direction for Abp1's functions in *Dictyostelium* besides its functions in pseudopodia formation during chemotaxis shown in the previous chapters.

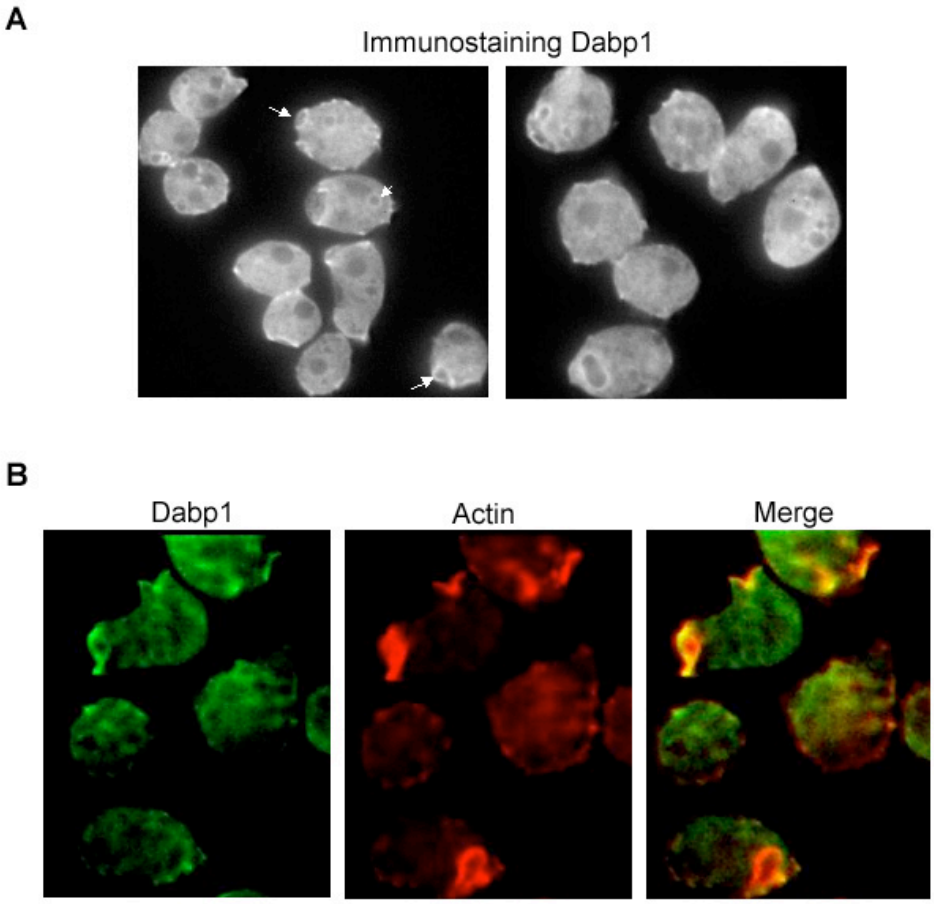

Figure 4.1 Dabp1 is associated with endocytic structures. (A) Dabp1 accumulates in crowns (endocytic cups) and vacuoles. Cells were fixed and stained with affinity-purified anti-Dabp1 antibodies followed by BODIPY FL-labeled secondary antibodies. (B) Dabp1 co-localizes with actin on crowns. Cells were fixed and stained with affinity-purified anti-Dabp1 antibodies followed by BODIPY FL-labeled secondary antibodies (to detect Dabp1) and TXRED-labeled phalloidin (to detect actin).

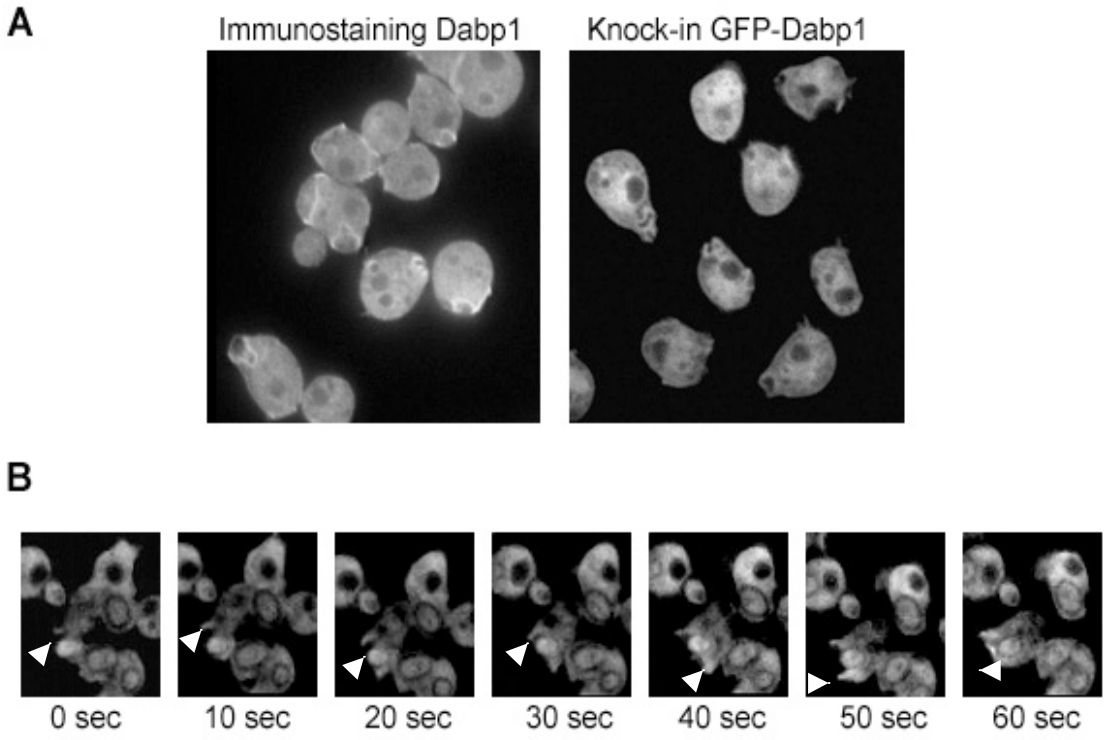

Figure 4.2 Dabp1 is associated with the formation of early endosomes. (A) GFP-Dabp1 showed similar localization as immunostaining Dabp1 in wild type cells. Fixed cells were stained with affinity-purified anti-Dabp1 antibodies followed by BODIPY FL-labeled secondary antibodies (upper panel, left). Cells expressing GFP-Dabp1 were fixed and examined under fluorescence microscope. (B) Dabp1 is involved in the early stage of endocytosis. Live cells expressing GFP-Dabp1 were incubated with heat-killed yeasts and imaged throughout phagocytosis.

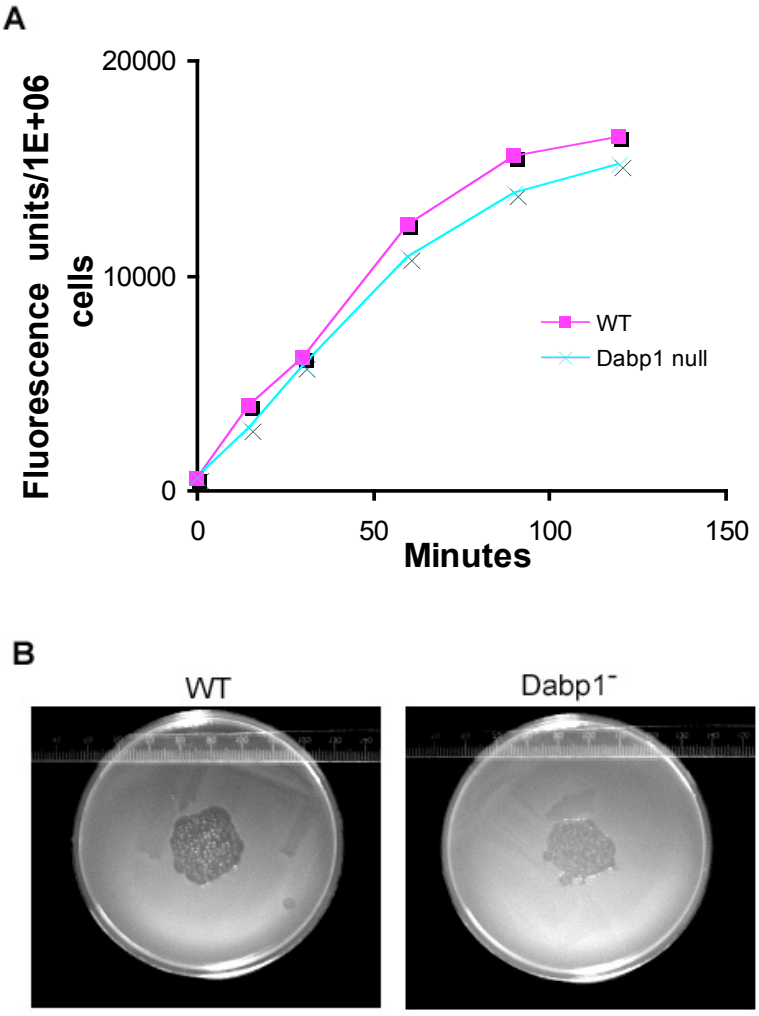

Figure 4.3 Deletion of Dabp1 had no effect on pinocytosis or phagocytosis on bacteria lawn. (A) Deletion of Dabp1 had no effect on pinocytosis. The uptake of FITC-dextran was measured during the course of 2h for wildtype cells (Ax2) and Dabp1$^-$ cells (B) Deletion of Dabp1 didn't affect phagocytosis on bacteria lawn. 2.5 x 10^4 of wildtype cells (Ax2) and Dabp1$^-$ cells were spotted on plates covered with *E.coli* bacteria lawn, respectively. Diameters of the growing circles on wildtype cell plates and Dabp1$^-$ cell plates were measured after 5 days incubation.

Chapter 5 Conclusions and Future Directions

Identification of a new Abp1 member in *Dictyostelium*

In this work I have identified and cloned the ortholog of Abp1 from *Dictyostelium*. The 481-amino acid Dabp1 is slightly larger than the 454-amino acid mAbp1, but smaller than the 591-amino acids yeast Abp1. Sequence analysis revealed that Dabp1 has an actin-binding ADFH domain at the N-terminus and an SH3 domain at the C-terminus that share high amino acid identity with similar domains in yeast Abp1. Like other members of the Abp1 family, Dabp1 also has a proline-rich region amino-terminal to the SH3 domain. These domains give Dabp1 the potential to link proteins via the SH3 or the polyproline domains, or to the actin cytoskeleton via the ADFH domain.

Immunofluorescence microscopy revealed that Dabp1 localizes to cell cortex, crowns and endocytic cups in vegetative cells and concentrates to the leading edge in developing cells. Yeast Abp1 is mainly localized to the actin patches while mAbp1 localizes to the perinuclear region, but upon activation of the GTPase Rac, mAbp1 translocated to the leading edge (Drubin et al., 1988; Kessels et al., 2000). The localization of Abp1 proteins suggests that they bind to sites of F-actin that are undergoing dynamic transitions.

Studies of Dabp1 reveal new functions for a member of the Abp1 protein family

1. Dabp1 functions in pseudopodia formation

When Dabp1 was eliminated from *Dictyostelium* cells by homologous recombination, most actin-based processes remained intact in these cells, such as cytokinesis, pinocytosis, or phagocytosis on a bacterial lawn. Deletion or overexpression of Dabp1 in

Dictyostelium cells had no effect on the actin cytoskeleton as determined by staining mutant cells with labeled phalloidin. In yeast cells, Abp1 enhances actin polymerization by acting as a weak activator for the Arp2/3 complex. However, deletion of Dabp1 in *Dictyostelium* cells caused an increased number of pseudopodia in chemotaxing cells during development. Overexpression of Dabp1 not only increased the pseudopodium number dramatically but also reduced the motility of chemotaxing cells (Wang and O'Halloran T, 2006). So far, Abp1 has not been found to function in chemotaxis in species other than *Dictyostelium*. Since yeast Abp1 and mAbp1 mainly function in endocytic pathway, studies of Dabp1 in pseudopodia formation revealed a new function for the Abp1 protein family (Fazi et al., 2002; Kessels et al., 2001).

2. Dabp1 is required by MyoB to regulate actin structures during chemotaxis

For efficient movement of streaming cells during chemotaxis, reorganization of the actin cytoskeleton is crucial. The formation of one dominant pseudopodium results from reorganization of the actin cytoskeleton and this activity needs to be finely regulated. Pseudopodia number, turnover of pseudopodia and a uniform shape of pseudopodia are three major aspects for the regulation of pseudopodia. Although Dabp1 was shown to function in pseudopodia formation, it was not clear how Dabp1 works in this process. By studying the relationship between Dabp1 and MyoB, I found that Dabp1 and MyoB work cooperatively to regulate the uniformity of pseudopodium shape. Deletion of both Dabp1 and MyoB dramatically reduces the stability of the leading edge and breaks the leading edge into many small branches. The uniformity and integrity of the pseudopodia are altered in these cells, which may alter the ability of this region to sense gradients in

chemotactic signals during chemotaxis. Since Dabp1 is important for the localization of MyoB, Dabp1 may function in pseudopodia formation by recruiting MyoB to the correct intracellular location, especially at the leading edge of chemotaxing cells.

The SH3 domain of Dabp1 is important for controlling pseudopodium number and overexpression of this SH3 domain rescues the defects caused by deletion of MyoB. Possibly, Dabp1 works with MyoB via its SH3 domain mediated interaction. The SH3 domains in yeast Abp1 and mAbp1 are also crucial to their functions in endocytosis. Taken together, the conserved SH3 domains for these members contribute to the diverse functions of Abp1 family.

Collectively, my work is the first to identify a role for Abp1 in regulating both the number and the uniformity of pseudopodia in *Dictyostelium*. My studies also provide evidence of how the uniformity of pseudopodium shape contributes to efficient movement during chemotaxis.

3. Dabp1 is associated with the formation of early endosomes during endocytosis

Like orthologs in other species, Dabp1 is also involved in endocytosis, especially the early stage of macropinocytosis and phagocytosis when early endosomes are formed. It is not clear how Dabp1 functions in this process. One explanation is that Dabp1 interacts with MyoK, a long-tailed myosin I known to function in phagocytosis and to maintain the cortical tension (Schwarz et al., 2000). Since MyoK binds Dabp1 via its GPA domain in the tail region, Dabp1 might work with MyoK during the initiation of phagocytosis (Soldati, 2003). Another possibility is Dabp1 acts as a scaffold to recruit MyoB, which functions in formation of membrane projections for phagocytosis or pinocytosis (Novak

et al., 1995). Dabp1 could also recruit proteins that regulate MyoK or MyoB, such as PAKs. Unlike other Abp1 members, which function in endocytosis by binding to Dynamin or other kinases implicated in endocytosis, Dabp1 is most likely involved in endocytosis by association with Myosin I proteins or regulators of Myosin I proteins. These studies of Dabp1 proposed a potential new mechanism for Abp1 members to function in endocytosis.

FUTURE DIRECTIONS

Isolation of the binding partners of Dabp1

In yeast, Abp1 binds to six ligands through its SH3 domain. All the six ligands are involved in endocytosis, which links yeast Abp1 to the endocytosis process (Fazi et al., 2002). Dabp1 shares high identity of SH3 domain with yeast Abp1 at the COOH terminus. The SH3 domain of Dabp1 is found to be essential for pseudopodia formation and the regulation of MyoB function. Isolation of binding partners of Dabp1, especially of the SH3 domain on Dabp1, will help to further explore Dabp1's functions during chemotaxis or other possible cellular processes.

Dabp1 was fused with TAP tag and transformed inside of *Dictyostelium* cells. TAP purification can be performed to isolate its binding partners (Puig et al., 2001). Also GST-Dabp1 was purified to generate anti-Dabp1 polyclonal antibodies in rabbits. These antibodies are very strong and affinity-purified anti-Dabp1 antibodies are very specific. Immunoprecipitation can be performed to isolate binding partners. By TAP purification or immunoprecipitation, whether Dabp1 binds MyoB directly or not can be confirmed.

Investigation of Dabp1's functions in the actin cytoskeleton

As an actin associated protein, Dabp1 is not required for organization of the actin cytoskeleton. It is not clear whether Dabp1 binds F-actin directly, although it has an actin-binding domain, the ADFH domain, at the N-terminus. Analysis of the ADFH domain will give a clue to this question. The sequence of Dabp1 also contains one acidic motif DDW, a motif with potential for binding and activating the Arp2/3 complex. It is known that yeast Abp1 functions as an activator of Arp2/3 because of the two DDW motifs contained in the sequence (Goode et al., 2001). It is possible that Dabp1 also functions as an activator for the Arp2/3 complex and thus is involved in the machinery for actin assembly. One piece of supportive evidence is that Dabp1 has extensive co-localization with the Arp2/3 complex in both vegetative cells and developing cells (data not shown).

Examination of the possible association between Dabp1 and MyoB

Dabp1 might recruit MyoB by binding directly to MyoB. If Dabp1 does not bind to MyoB directly, it is also possible that Dabp1 recruits the regulators of MyoB and thus affects the functions of MyoB. For example, PAK members function to regulate the phosphorylation of Myosin I proteins or Myosin II, and play a role in pseudopod formation and cell movement during chemotaxis (Chung and Firtel, 1999; Chung et al., 2001; Lee et al., 2004). The N-terminal regulatory domain of several PAKs have proline-rich sequences that can mediate interactions with SH3 domain-containing proteins (Bagrodia and Cerione, 1999). Dabp1 could bind to these regulators via its SH3 domain and then influence the regulation of Myosin Is, such as MyoB.

PAK family includes MIHCK, PAKa, PAKb and PAKc. Since PAKa mainly functions to phosphorylate Myosin II, MIHCK selectively phosphorylates MyoD and PAKb mainly functions in pinocytosis or phagocytosis, PAKc is a potential regulator for the phosphorylation activity of MyoB during chemotaxis (Chung and Firtel, 1999; Chung et al., 2001; de la Roche et al., 2005; de la Roche and Cote, 2001; Lee and Cote, 1995). To test whether Dabp1 affects the functions of MyoB by recruiting the regulators of MyoB, the localization of PAKc could be investigated in both wildtype cells and Dabp1$^-$ cells.

Quantification of velocity in Dabp1$^-$ /MyoB$^-$ cells during chemotaxis

MyoB$^-$ cells have reduced velocity while Dabp1$^-$ cells exhibits almost no defects in motility. Since both mutants extend increased pseudopodia during chemotaxis, pseudopodium number might not be the only aspect required for efficient movement (Wang and O'Halloran T, 2006; Wessels et al., 1991). Since the integrity of cellular extensions is required for proper pattern of dentritic fields in *Drosophila*, regulation of integrity of actin structures is important for their functions (Cong et al., 2001; Emoto et al., 2004). Maintenance of the uniformity and integrity of pseudopodia might also be important for efficient movement in chemotaxing *Dictyostelium* cells. In Chapter 3, I have shown that developing Dabp1$^-$/MyoB$^-$ cells fail to maintain the uniform pseudopodium shape and break into many small branches. Studies of the motility in Dabp1$^-$ /MyoB$^-$ cells would help to illuminate the relationship between misregulated pseudopodia and cell movement during chemotaxis. In these mutants, the number and the

shape of pseudopodia are both altered. Both aspects might be important for the functions of pseudopodia and need to be tightly controlled.

Quantification of pinocytosis and phagocytosis in Dabp1$^-$/MyoB$^-$ cells

MyoB was known to function at the early stage of phagocytosis or pinocytosis although it is not essential for these processes (de la Roche and Cote, 2001). Here we have shown that Dabp1 was also associated with early endosomes. As I hypothesized earlier, Dabp1 might function as a scaffold to recruit long-tailed myosin I proteins, such as MyoB. Although Dabp1 or MyoB single null mutants had no defects in pinocytosis or phagocytosis, depletion of both Dabp1 and MyoB could influence cortical tension and then alter the organization of actin cortex. In support of this is our observation that Dabp1$^-$/MyoB$^-$ cells grew slower in suspension (data not shown). It is possible that the Dabp1$^-$/MyoB$^-$ cells have defects in pinocytosis similar to MyoB$^-$/MyoC$^-$ mutants or MyoA$^-$/MyoB$^-$ mutants (Novak et al., 1995). To test this possibility, the uptake of FITC-dextran can be quantified in the Dabp1$^-$/MyoB$^-$ cells. Similarly, to test whether Dabp1$^-$/MyoB$^-$ cells have defects in phagocytosis, double null cells can be plated on bacteria lawns and the growing circles on the plates can be measured as described previously in Chapter 4.

Examination of the vesicles (early endosomes) in Dabp1$^-$/MyoB$^-$ cells during endocytosis

Early endosomes are formed by the extension of cell cortex to engulf cargo and bring it into the cell. The newly formed endosomes are coated with two layers of proteins: the inside layer is coronin while the outside layer is actin (Maniak, 2003). Deletion of both

Dabp1 and MyoB could affect the rearrangement of the actin cytoskeleton and thereby affect the actin layers on early endosomes. In Chapter 3, Dabp1$^-$ /MyoB$^-$ cells were shown to have an altered cytoskeleton even during growth, and were covered with F-actin filled spikes on their cell bodies. A possible alteration of the actin cytoskeleton could be tested directly by looking at the localization of actin-binding proteins such as coronin in Dabp1$^-$/MyoB$^-$ cells. Fluorescent FITC-dextran can be also be used to investigate the size or number of vesicles in these double null cells.

Chapter 6 Experimental Procedures

Part I: Cell lines and Cell Culture

Table 6.1 Miscellaneous cell lines

Dictyostelium cell lines used in Chapter 2	WT (wildtype) Ax2 Dabp1$^-$ (Dabp1 null) cells generated from Ax2 Dabp1$^+$ cells (Ax2 cells overexpressing Dabp1)
Dictyostelium cell lines used in Chapter 3	WT (wild type) DH1 cells MyoB$^-$ (MyoB null) cells generated from DH1 Dabp1$^-$ (Dabp1 null) cells generated from DH1 Dabp1$^-$/MyoB$^-$ (MyoB and Dabp1 double null) cells WT (DH1) expressing MyoB Dabp1$^-$ cells expressing MyoB WT (DH1) expressing Dabp1 MyoB$^-$ cells expressing Dabp1
Dictyostelium cell lines used in Chapter 4	WT (wild type) Ax2 Dabp1$^-$ (Dabp1 null) cells generated from Ax2 WT cells expressing integrating GFP-Dabp1

DH1 cells and MyoB$^-$ cells are gifts from Dr. Margaret Titus at University of Minnesota. The generation of MyoB$^-$ cells was described in their previous studies (Novak et al., 1995)

All cell lines were maintained on Petri dishes at 18^{o}C. Ax2 cells, MyoB$^-$ cells and DH1 cells were cultured in HL-5 media. Two different Dabp1$^-$ cell lines and Dabp1$^-$

/MyoB$^-$ cells were cultured in HL-5 with 5 µg/ml Blasticidin (ICN, Biomedicals Inc). Ax2 cells expressing Dabp1, MyoB$^-$ expressing Dabp1 and DH1 cells expressing MyoB were maintained in HL-5 with 10 µg/ml G418 (Geneticin; GIBCO BRL). Dabp1$^-$ cells expressing MyoB were grown in HL-5 with both 10 µg/ml G418 and 5 µg/ml Blasticidin.

Part II: Plasmids and Plasmid constructions

cDNA cloning and sequence analysis

The DNA sequence of yeast *Abp1* (accession number X51780) was used to search the *Dictyostelium* genome database (http://www.dictybase.org) for the best match using reciprocal BLAST. A single gene ortholog was obtained, *Dictyostelium abpE*. A second database, InParanoid (http://inparanoid.cgb.ki.se) searched with BLAST also identified the *abpE* gene as the Dictyostelium abp1 ortholog. A complete cDNA clone was obtained from a cDNA library using a Polymerase Chain Reaction (PCR) -based strategy using primers 5' CCGGATCCATGGCATCATTAGATATTAGTGATCCAGATATTAC 3' and 5'CCGAATTCCTCGAGTTACAATTGTTGTACGAAATTAGATGGGAAG3'. Predicted protein sequences were analyzed using program Megalign (DNAStar, Inc., Madison, WI) with the default ClustalV parameters.

Plasmid used to create Dabp1 null cell lines

This vector (named as psP72-Dabp1) was constructed by first using PCR to amplify sequences corresponding to regions that flanked the 5' and the 3' ends of the entire coding sequence for the *abpE* gene. The primers 5' GAGCTCTTGTAGTTCCCCTTACCAAATCATTGTG 3' and 5'

GGATCCGTTTTGGTTCAAAGAATAATATTTGTTGG 3' were used to amplify a 5' fragment flanked by SacI and BamHI sties. The primers 5' AAGCTTAACTATTTCCATTTGTTTTCCTTATTTATCC 3' and 5' CTCGAGAGGGGTGTCTCTGGCTGTGTCG 3' were used to amplify a 3' fragment flanked by HindIII and XhoI sites. Both fragments were subsequently cloned into the plasmid pSP72-BSR (Wang et al., 2002) so that the blasticidin gene was flanked by these two fragments.

Plasmid used to purify Dabp1 protein

A cDNA for Dabp1 was cloned into the BamHI and EcoRI sites of the plasmid PGEX-2T. In this plasmid (PGEX-Dabp1), the cDNA for Dabp1 was inserted downstream of the glutathione-S-transferase (GST) gene, resulting in expression of a GST-Dabp1 fusion protein.

Plasmid used to express Dabp1, SH3 domain of Dabp1 and ADFH domain of Dabp1

To make a construct for Dabp1 expression (pTX-GFP-Dabp1), a cDNA for Dabp1 expression was PCR-amplified from a *Dictyostelium* cDNA library with primers 3' CCGAATTCCTCG AGTTACAATTGTTGTACGAAATTAGATGGGAAG 5' and 5' GGATCCG CAGCAGCAGCAGCAATGGCATCATTAGATATTAGTGATCCAGATATTAC 3'. This fragment was cloned into the BamHI and XhoI sites of the plasmid pTX-GFP (Levi et al., 2000), placing Green Fluorescent protein (GFP) at the amino-terminus of the Dabp1 protein. A linker containing five alanines was included between GFP and Dabp1.

To make an expression vector for Dabp1 without the GFP tag (pTX-Dabp1), a modification was made to the pTX-GFP-Dabp1 plasmid by removing the GFP cassette at the BamHI and HindIII sites, blunting and religating the plasmid.

The ADFH and SH3 domains of Dabp1 were identified by comparing the sequence of Dabp1 with conserved domains using NCBI software (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). To make an expression vector for the ADFH domain (pTX-GFP-Dabp1/ADFH), the first 130 amino acids of Dabp1 were cloned by PCR from a *Dictyostelium* cDNA library. This sequence was cloned into the pTX-GFP plasmid with GFP placed at the amino-terminus of the ADFH domain. A linker of five alanines separated the GFP and the ADFH domain.

To make an expression vector for the SH3 domain (pTX-GFP-Dabp1/SH3), the carboxyl-terminal 69 amino acids of Dabp1, (the 59 amino acids of the entire SH3 domain and 10 additional amino acids amino-terminal to the SH3 domain) were amplified by PCR from a *Dictyostelium* cDNA library. This DNA fragment was cloned into the pTX-GFP plasmid with GFP fused at the amino-terminus of the SH3 domain.

Plasmid used to express integrating GFP-Dabp1

To create a vector, pSP72-int Dabp1, for the integration of GFP-Dabp1 into the genome, GFP-Dabp1 fragment was excised from pTX-GFP-Dabp1 plasmid by digesting at both SalI and XhoI sites. psP72-Dabp1 plasmid made to generate Dabp1 null cells was digested by HindIII and XhoI to remove 3' UTR. Linerized psp72-Dabp1 plasmid without 3'UTR was ligated with GFP-Dabp1 fragment by XhoI ligation at one end plus HindIII and SalI ligation at the other end.

Plasmid used to express GFP-MyoB

A gift from Dr. Margaret Titus (University of Minnesota), the construction of the expression plasmid for GFP-MyoB, pDT b69, was described in their previous studies (Novak et al., 1995).

Table 6.2 Plasmids and their functions

Plasmid	Functions
pSP72-Dabp1	replace *AbpE* gene, generate Dabp1 null cells
pSP72-int Dabp1	integrate GFP-Dabp1 gene into genome
pTX-GFP-Dabp1	overexpress GFP tagged Dabp1
pTX-Dabp1	overexpress Dabp1
pTX-GFP-Dabp1/SH3	overexpress GFP tagged SH3 domain
pTX-GFP-Dabp1/ADFH	overexpress GFP tagged ADFH domain
PGEX-Dabp1	express GST tagged Dabp1 for protein purification
pDTb69	express GFP-MyoB

Part III: *Dictyostelium* Transformations

The transformation of *Dictyostelium* was performed according to a protocol described in previous studies (Kuspa and Loomis, 1992).

Generation of Dabp1 null cells from Ax2 or DH1 background and generation of Dabp1/MyoB double null cells

A linear DNA fragment containing this *abpE* gene replacement cassette was excised using the flanking SacI and XhoI restriction sites of plasmid psP72-Dabp1. After treating with phosphatase, 5 μg of this linear DNA fragment was used to transform by electroporation into Ax2 cells or DH1 cells to generate Dabp1$^-$ cells. The same fragment was used to transform MyoB$^-$ cells to create Dabp1$^-$/MyoB$^-$ cells. After transformation, cells were diluted into 96-well plates, and cultured with blasticidin to generate clonal transformants. Clonal transformants in which the entire coding region of Dabp1 was replaced by integration of the blasticidin marker were identified using PCR analysis. The absence of Dabp1 expression was confirmed in these cells by western blot analysis with anti-Dabp1 antibodies.

Generation of cells expressing integrating Dabp1

A linear DNA fragment containing 5'UTR-Blasticitin marker-GFP Dabp1 was removed from pSP72-int Dabp1 plasmid by digesting with enzymes ClaI and XhoI. 5 μg of this linear DNA fragment was transformed into Ax2 cells. Transformed cells were diluted into 96-well plates, and cultured with blasticidin to select clonal transformants. The expression level of GFP-Dabp1 was examined in these transformant by western blot analysis with anti-Dabp1 antibodies.

Generation of cells expressing Dabp1, SH3 domain or ADFH domain

pTX-GFP-Dabp1 plasmid and pTX-Dabp1 plasmid were used to transform Ax2 cells to create cells expressing either GFP tagged Dabp1 or Dabp1. Clonal transformants were selected by culturing transformed cells in media with G418. The expression level of Dabp1 was examined in these cells by western blot analysis with anti-Dabp1 antibodies.

To express Dabp1 in NC DH1 cells and MyoB null cells, pTX-GFP-Dabp1 plasmid was transformed into NC DH1 and MyoB null cells. Transformants were selected by adding G418 to the culture. The expression level of Dabp1 in these cells was confirmed as described above.

To express ADFH domain in Ax2 cells or MyoB null cells, pTX-GFP-Dabp1/ADFH plasmid was transformed into Ax2 cells or MyoB nulls. Transformed cells were cultured in media with G418 to select transformants. The expression level of ADFH domain was examined by western blot analysis with anti-Dabp1 antibodies. The expression of SH3 domain in Ax2 cells or MyoB null cells was performed the same as ADFH domain by using plasmid pTX-GFP-Dabp1/SH3.

Generation of cells expressing MyoB

To express MyoB in DH1 cells or Dabp1 null cells generated from DH1 background, pDTb69 plasmid was transformed into these two cell lines. G418 was used to select transformants from transformed DH1 cells while G418 and blasticidin were used to select transformants from transformed Dabp1 null cells. The expression level of MyoB was compared by western blot analysis with anti-MyoB antibodies (anti-MyoB antibodies were donated by Dr. Margaret Titus at University of Minnesota).

Part IV: Miscellaneous Assays

Assays for Chapter 2

Protein purification and generation of antibodies to Dabp1

PGEX-Dabp1 plasmid was transformed into *Escherichia coli* DH5α for large-scale protein purification. To purify the GST-Dabp1 fusion protein, cell cultures and cell lysates were prepared as described previously (Vithalani et al., 1998). The purified GST-Dabp1 protein was used to generate polyclonal anti-Dabp1 antibodies in rabbits (Cocalico Biologicals, Reamstown, PA). The polyclonal anti-Dabp1 antibodies were affinity-purified on a GST column using a GST orientation kit (PIERCE, Rockford, IL).

Immunofluorescence microscopy

Ax2, Dabp1$^-$ and Dabp1$^+$ Cells were harvested at middle-log phase, adjusted to 2 x 10^6 cells/ml and allowed to settle on glass coverslips for 20 minutes at 18^0C. Cells were fixed for 5 minutes at -20^0C by incubation with methanol containing 1% formaldehyde. To improve imaging, some cells were gently flattened with a square of 2% agar NA (Amersham Biosciences, Uppsala, Sweden) before fixing for 5 minutes in methanol containing 1% formaldehyde at -20^0C (Fukui et al., 1987). Subsequently, fixed cells were processed for immunostaining.

To immunostain, affinity-purified anti-Dabp1 antibodies (15 µg/ml) were incubated with fixed cells on coverslips at 37^0C for 40 minutes. After washing coverslips four times with phosphate-buffed saline (PBS), BODIPY FL-conjugated goat-anti-rabbit IgG (30 µg/ml, Molecular Probes, Eugene, OR) was incubated with each coverslip at 37^0C for 40 minutes. To detect actin, fixed cells coverslips incubated with TXRED-labeled phalloidin (0.3 unit/ml, Molecular Probes, Eugene, OR).

For cytochalasin treatment, cells adhered to coverslips were incubated with 10 µM cytochalasin A at room temperature. After 30-60 min, cells were flattened and fixed for 5

minutes in methanol with 1% formaldehyde at -20^{0}C. For double-labeling both Dabp1 and actin, immunostaining with anti-Dabp1 antibodies was followed by TXRED-labeled phalloidin. Images were taken on an inverted NIKON microscope TE200 (NIKON instruments, Dallas, TX) with 100x 1.4NA PlanFluor objective and Quantix 57 camera (Roper Scientific. AZ) controlled by Metamorph (Universal Image Corp., PA), and then processed using Adobe Photoshop software.

Light microscopy

For studying the morphology of streaming Ax2, Dabp1$^-$ and Dabp1$^+$ cells, cells were imaged on an inverted NIKON microscope TE200 with either the 20x objective or the 100x objective. For studying development, images were captured on a Zeiss microscope Semi SR with 0.8x or 1.2x objectives controlled by NIH image software.

Velocity measurement assays

To study the motility of streaming Ax2, Dabp1$^-$ and Dabp1$^+$ cells, cells were harvested at late-log phase, washed once with the starvation buffer, PDF (2 mM KCL, 1.1 mM K_2HPO4, 1.32 mM KH_2PO4, 0.1 mM $CaCL_2$, 0.25 mM MgSO4, pH=6.7), and then resuspended into the same PDF buffer at a density of 2x10^6 cells/ml. 400 µl of the cell suspension were added to a one-well coverslip-chamber (Nulge-Nunc Int., Naperville, IL) and incubated at 18^{0}C until cells were actively streaming. Images of cells moving toward aggregation centers were recorded at 6-second intervals using an inverted NIKON microscope TE200 (NIKON instruments, Dallas, TX) with a 60x 1.4NA PlanFluor objective and a Quantix 57 camera (Roper Scientific, AZ) controlled by Metamorph (Universal Image Corp., PA).

The velocity of streaming cells was measured by studying the movement of a single cell outside of the aggregation center in the images of moving cells. The longest extension of the uropod of the streaming cell was used as a starting point. The x and y position of the uropod in each frame was noted. The distance moved by the tail at a given time was calculated from the z value derived from the x and y values ($z^2=x^2+y^2$) and converted from pixel to μm (1 μm= 4.52 pixel). Velocity was calculated from the z value and time (v=z/t).

Streaming and development assays

To study the localization of Dabp1 or actin in the starved cells undergoing chemotactic aggregation, cells were harvested at late-log phase, washed once with PDF, and then resuspended at a density of $2x10^6$ cells/ml in PDF buffer. 200 μl cell suspension was spotted on a coverslip and incubated in a humidified chamber at 18^0C until cells were actively streaming. Cells were then gently flattened with an agarose square, then fixed and stained with affinity-purified anti-Dabp1 antibodies or TXRED-labeled phalloidin as described above.

To study the development of starved cells on agar plates, $1x10^8$ of Ax2, Dabp1$^-$ or Dabp1$^+$ cells were harvested at late-log phase. Cells were washed once with PDF and resuspended into 3 ml of PDF. The cell suspension was spread on a PDF agar plate (20 g agar/1L PDF, 30 ml/plate, prepared the day before use) and allowed to settle for 40 minutes at room temperature. After aspirating excess liquid, cells were allowed to develop at 18^0C for 30 hrs. Images were taken with a Zeiss microscope Semi SR with 0.8x or 1.2x objectives controlled by NIH image software. Western blot analysis

demonstrated an equivalent amount of Dabp1 protein throughout development in wildtype and in Dabp1^{+} cells (data not shown).

Quantification of pseudopodium number

Developing Ax2, SH3^{+}, ADFH^{+}, Dabp1^{-} and Dabp1^{+} cells were prepared and imaged as described in velocity measurement assays. For Dabp1^{-} cells, three independent cell lines were examined. For each cell line, approximately 80-100 cells were selected and an outline of the cell perimeter was drawn. Protrusions extending from the cell body were counted as pseudopodia.

Assays for Chapter 3

Immunofluorescence microscopy

To immunostain Dabp1 in DH1 cells expressing GFP-MyoB, cells were prepared and imaged as described in assays for chapter 2. Fixed cells were incubated with 100 µl of the affinity-purified anti-Dabp1 antibodies to detect Dabp1.

To examine the localization of F-actin in wildtype, Dabp1^{-} cells, MyoB^{-} cells and Dabp1^{-}/MyoB^{-} cells, Texas red labeled phalloidin was used to detect F-actin as described in assays for Chapter 2.

To observe GFP-MyoB in wildtype expressing GFP-MyoB or Dabp1^{-} cells expressing GFP-MyoB, cells were allowed to attach on the coverslips and fixed for 5 minutes at -20^{0}C by incubation with methanol containing 1% formaldehyde. Images were taken as described in assays for Chapter 2.

Triton cytoskeleton assays

2 x 10^7 of wildtype, Dabp1$^-$ cells and MyoB$^-$ cells were harvested and pelleted at 1000g for 3 minutes. Pellet was washed once with 1ml cold 100 mM MES (PH 6.8) and resuspended into 1ml ice-cold lysis buffer (100 mM MES PH6.8, 1 mM $Mgcl_2$, 0.5 % Triton X-100, 2.5 mM EGTA) with the cocktail of protein inhibitors (1 mM DTT, 5 μg/ml Leupeptin, 0.1 mM PMSF, 1.4 μg/ml Pepestatin A). The suspension was spun down at 14000 g for 10 seconds. Subsequently, the supernatant was transferred to the chilled tubes without disturbing the pellet. The triton insoluble pellet was gently washed with 1 ml cold lysis buffer (this is to minimize the contamination of the supernatant). Then 1 ml lysis buffer plus protein inhibitor described as above was added to resuspend the washed pellet. All steps in the assays were performed at 4^0C. Equivalent volumes were taken from the supernatant and the triton insoluble pellet in each cell line and loaded on SDS-PAGE gel for western blots. A duplicate gel was also made for coomassie stain in each cell line.

Streaming and development assays

To study the morphology of Dabp1$^-$/MyoB$^-$ cells, MyoB$^-$ cells expressing Dabp1 or Dabp1$^-$ cells expressing MyoB during chemotaxis, cells were prepared and imaged as described in assays for Chapter 2.

Quantification of protrusions

Developing DH1 cells, MyoB$^-$ cells, Dabp1$^-$ cells, Dabp1$^-$/MyoB$^-$ cells, DH1 cells expressing MyoB, Dabp1$^-$ cells expressing MyoB, DH1 cells expressing Dabp1, MyoB$^-$ cells expressing Dabp1, MyoB$^-$ cells expressing ADFH domain and MyoB$^-$ cells

expressing SH3 domain were prepared and imaged as described in Chapter 2. Cell perimeters were outlined for approximately 200 cells for each cell line, each selected from three independent experiments. Protrusions extending from the cell body were counted.

Assays for Chapter 4

Immunofluorescence microscopy

To double stain Dabp1 and F-actin in Ax2 cells, cells were first harvested and attached on coverslips. Before fixing, cells weren't flattened in order to keep maintain native structures. Fixation and staining with affinity-purified Dabp1 antibodies followed by TEXAS red-labeled phalloidin were performed the same as described in assays for Chapter 2.

Pinocytosis assay

Pinocytosis assays of Ax2 cells and Dabp1$^-$ cells were carried out by using FITC-Dextran as described in previous studies (Klein and Satre, 1986). Samples from each cell line were collected at 0, 15min, 30min, 60min, 90min and 120min during 2-hour time course. Fluorescence intensities were measured using excitation and emission wavelengths of 485 and 520 nm, respectively.

Yeast phagocytosis assay

5 x 10^6 Ax2 cells expressing integrated GFP-Dabp1 were harvested at mid-log stage, washed with PB, resuspended into PB and then allowed to attach in two–well coverslip-chamber (Nulge-Nunc Int., Naperville, IL) for 20 min. Around 5 x 10^7 heat-killed yeast were added to the chamber, incubated for 10 minutes and processed for fluorescence

microscopy. Cell starting to intake yeasts were chosen and images were taken every 10 seconds to record the intake process on an inverted NIKON microscope TE200 under 100x objective.

Phagocytosis assay on bacteria lawn

2 ml B/R *E.coli* was grown in plain HL-5 media overnight at 37^0C. 200 µl of the overnight *E.coli* culture was plated evenly on SM/5 plates (2 g proteose peptone #2/L, 2 g yeast extract/L, 2 g glucose/L, 0.2 g MgSO4/L, 1.9 g KH_2PO4/L, 1 g K_2HPO4/L, 20 g agar/L) and then incubated at 37^0C overnight to obtain bacteria lawns. Ax2 cells and Dabp1$^-$ cells were harvested at mid-log stage. 25 µl of $1x10^6$ cells/ml culture from each cell line was spotted in the middle of SM/5 plates with bacteria lawn and then incubated at 18^0C. The growing circle on each plated were imaged and measured every two days.

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Vita

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